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A
Thesis

Presented to the Faculty of
The University of Texas Graduate School of Biomedical Sciences
at San Antonio
in Partial Fulfillment
of the Requirements
for the Degree of

MASTER OF SCIENCE

By
Alan John Moritz, B.S., D.D.S.

San Antonio, Texas

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DEDICATION

This thesis would not have been possible without the steadfast love, support, and understanding of my beautiful wife Betsy. It was through her numerous sacrifices and constant reassurances that I was able to concentrate my energies on successfully fulfilling the requirements of a very demanding periodontics residency as well as a very satisfying research project. For this I will always be grateful to her. To my son Stephan and my daughter Julia; I hope that the perseverance and dedication that I have demonstrated in my present educational endeavors encourages each of you to pursue your dreams in a similarly relentless fashion. The sacrifices each of you made for me I hope to repay to you many times over in the coming years. To my parents, Hans and Christa Moritz who from the very start instilled within me a burning desire to pursue knowledge and to be the best I can be.

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IMMUNIZATION WITH *PORPHYROMONAS GINGIVALIS* CYSTEINE PROTEASE: EFFECTS ON EXPERIMENTAL GINGIVITIS AND LIGATURE-INDUCED PERIODONTITIS IN *MACACA FASCICULARIS*

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Targeting specific bacterial virulence factors such as proteases for immunization may hold the key to limiting or preventing loss of attachment and alveolar bone in periodontal disease. This study was undertaken to examine the clinical, microbiological and immunological responses following active immunization with a purified 120 kDa *Porphyromonas gingivalis* cysteine protease (porphypain-2) in the nonhuman primate (Nhp) *Macaca fascicularis* (cynomolgus monkey). 4 Nhp were immunized with 100μg of porphypain-2 antigen at baseline, 2 and 4 weeks. A "non-immunized" control group of 4 Nhp received placebo injections. The animals were subjected to a 4 week experimental gingivitis phase initiated by soft diet, followed by a 13 week ligature-induced periodontitis phase. Mandibular left "test" teeth (3-5, 3-6, 3-7) in each animal received silk ligatures to initiate periodontitis, with mandibular right teeth (4-5, 4-6, 4-7) in each animal serving as a non-ligated intra-animal control. Clinical, radiographic, microbiologic and immunologic data were gathered and analyzed using the student *t*-test and nonparametric rank analyses. An ELISA demonstrated that immunization elicited an elevated

and specific IgG response to both whole cell *P. gingivalis* (36-fold) and to porphypain-2 (194-fold). This antibody response in immunized Nhp remained significantly elevated versus baseline ($P < 0.0001$) and the control group ($P < 0.00001$) throughout the study. Checkerboard DNA probe analysis of 16 subgingival plaque species from ligated sextants demonstrated that 25% more gram-negative anaerobic species became significantly elevated from baseline and at earlier timepoints in the control group than in the immunized group. Immunization did not suppress the emergence of *P. gingivalis.* Clinical indices showed few changes related to immunization. CADIA assessment of bone density changes demonstrated a highly significant loss in ligated sextants compared to non-ligated sextants within the control group ($P < 0.001$), with a less significant difference within the immunized group ($P = 0.043$). Comparison of ligated sextants only, demonstrated more bone loss in the control group vs. the immunized group (-13.07 ± 9.51 vs. -9.41 ± 6.18; CADIA units ± SD); the difference approached but did not reach significance. While the *in vivo* mechanism(s) by which an immune response to porphypain-2 brought about the changes in the microbial ecology of the subgingival environment is unknown, the results suggest that this cysteine protease of *P. gingivalis* may be important in determining the pathogenic potential of the subgingival plaque microbiota in the Nhp model of ligature-induced periodontitis. Active immunization with porphypain-2 may be capable of altering this pathogenic response.

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1. Treatment Approaches

Adult periodontitis, far from being a predictably curable disease, continues to challenge dental clinicians from both etiologic and treatment standpoints. Left untreated, it may progress to eventual tooth loss. Short of tooth loss, morbidity in the form of alveolar bone loss, connective tissue attachment loss, gingival recession, bleeding, suppuration, and tooth mobility may result.

The means presently available for managing adult periodontitis revolve primarily around either non-specific disease prevention through bacterial plaque control, or in attempting to arrest, eliminate, or regenerate periodontal lesions after irreversible destruction has already occurred. Prevention prior to disease initiation and subsequent destruction of the periodontal attachment apparatus is obviously the preferred method. For this approach, the clinician relies heavily upon patient at-home oral hygiene measures and professionally-applied preventive maintenance along with early detection.

Managing and treating periodontal disease after destruction of the periodontium has taken place presents a vast challenge and entails either attempting to surgically eliminate or regenerate periodontal defects, or non-surgically maintaining periodontal pockets in order to forestall further disease progression. Regardless of the mode of therapy, the patient is ultimately placed in a maintenance recall program for supportive periodontal treatment. The

end result of these definitive periodontal procedures is often a reduced periodontium that exhibits treatment-related gingival recession with longer, more difficult to maintain teeth with exposed root surfaces that are subject to thermal sensitivity and carious attack.

Many treatment-limiting variables exist in the management approaches to adult periodontitis. If prevention of initial disease occurrence or disease recurrence post-treatment is to succeed, patient attitudes towards treatment, oral hygiene performance, and recall compliance are of paramount importance. Often patient motivation is directly related to frequency of recall. The more frequent and intense the oral hygiene instruction, the better compliance with oral hygiene that is achieved. Lindhe *et al.*, (1982) have shown that the level of oral hygiene established during maintenance is more important to success of the therapy than the actual mode of therapy used. Yet Wilson *et al.,* (1993) noted that even with intense efforts by dental personnel to motivate patients, only 32% were found to be complete compliers with suggested recall intervals.

The concept of prevention also implies early detection. At present, the clinical "gold standard" of longitudinal monitoring of attachment levels to discern disease activity is a less than ideal method of detecting incipient disease progression due primarily to the limitations inherent in periodontal probing. Recent advances in controlled-force probes may aid in this area of disease activity detection.

Additional limitations exist in the definitive treatment of the lesions of periodontal destruction. Although major strides in regenerative capabilities have been made in recent years, predictably and completely regenerating periodontal defects continues to be an elusive

pursuit. And even when treatment is expertly and effectively rendered, some cases of periodontitis appear refractory to treatment.

Ultimately economic concerns arise during the discussion of periodontal treatment alternatives. Periodontal therapy costs not only add to the overly burdensome costs of health care in our society, but also prevent many who would benefit from periodontal treatment from gaining access to care.

The failure to conquer adult periodontal disease is related to the inherent nature of the disease. Far from being just another bacterial infection, it has been repeatedly shown to be an extremely enigmatic disease process involving complex interactions between multiple bacterial species in the periodontal pocket, systemic and local host immune responses, and various extrinsic and intrinsic modifying factors. It is fair to say that our understanding of these bacterial-host interactions is in an extremely early stage in relation to our ultimately hoped for "cure" for this disease.

2. Clinical Characteristics

Human adult periodontitis is characterized by a destructive inflammatory lesion that ultimately causes loss of the connective tissue attachment to the tooth and alveolar bone destruction. It normally occurs in the mid thirties and arises in persons who are otherwise systemically healthy. Although originally believed to progress slowly, and unrelentingly with increasing age to result in inevitable tooth loss, destructive disease activity probably does have some continuously progressive component as well as random site-specific

destruction with exacerbations followed by periods of disease remission. Untreated adult periodontitis studies by Lindhe *et al.*, (1983) and Haffajee *et al.*, (1983) have shown that most disease progression occurs in a limited number of sites in a small number of individuals, and not at all in others.

The U.S. prevalence of adult periodontitis has been examined by cross-sectional epidemiological studies. Prevalence estimates range from nearly 100% of adults reported by Marshall-Day *et al.*, (1955) to 13.4% by Brown *et al.*, (1990). A more recent large scale study by Brown *et al.*, (1996) reported a prevalence of 29.2%. All studies to date are complicated by shortcomings in the various methods of clinical sampling employed, and the fact that the distinction is not made between the various forms of periodontal diseases.

3. Microbiological Characteristics

It is currently believed that microorganisms comprising the bacterial plaque are the primary etiologic factors in initiating adult periodontitis. The development of the destructive lesion of adult periodontitis is accompanied by a shift in the predominantly Gram-positive subgingival microbiota associated with periodontal health, to a highly pathogenic Gram-negative, anaerobic one.

Löe *et al.*, (1965) and Theilade *et al.*, (1967) demonstrated a distinct association between bacterial plaque accumulation and the subsequent development of experimental gingivitis in humans. Plaque associated with clinically healthy gingiva consists primarily of Gram-positive facultative species, predominantly *Streptococci* and *Actinomyces* which

constitute up to 85% of the total cultivable microbiota (Slots, 1979). Gingivitis has been associated with a shift from a *Streptococcus* dominated plaque to a more complex *Actinomyces* dominated plaque including increased numbers of Gram-negative and motile bacteria (Loesche and Syed, 1978; Listgarten and Hellden, 1978). Slots *et al.*, (1978) have shown the microbiota in human chronic gingivitis to be comprised of approximately 45% Gram-negative and 45% anaerobic species. The microbial composition of established periodontitis lesions demonstrates a further shift towards a more Gram-negative anaerobic plaque dominated by motile forms and rods (Listgarten and Hellden, 1978; Tanner *et al.*, 1979). Slots, (1977) showed the bacterial composition in advanced adult periodontitis lesions to be approximately 75% Gram-negative and 90% anaerobic species.

Nevertheless, a positive cause and effect relationship between putative periodontopathogenic species and the initiation of periodontal disease has not yet been established. Also, data concerning the progression of gingivitis to periodontitis is scarce in humans. Current evidence suggests that microbial pathogens are necessary, although not sufficient to cause periodontal disease. Of the more than 300 bacterial species that inhabit the oral cavity, only a handful are currently regarded as suspected periodontopathogens (Bragd *et al.*, 1987; Dzink *et al.*, 1988; Slots, 1986; Tanner *et al.*, 1987; Zambon *et al.*, 1983). Socransky and Haffajee, (1992) have identified the following species as having the most evidence for association with destructive periodontal diseases: *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Prevotella intermedia, Fusobacterium nucleatum, Bacteroides forsythus, Campylobacter rectus, Eikenella corrodens, Peptostreptococcus micros, Selenomonas sp., Eubacterium sp.*, and spirochetes. Of these, *P.*

gingivalis stands out as having an abundance of data to support its role as a possible etiologic agent in adult periodontitis.

a. *Porphyromonas gingivalis* in Periodontal Disease

P. gingivalis is a Gram-negative, nonmotile, obligately anaerobic rod shaped bacterium that has been repeatedly and ubiquitously identified as being associated with periodontal disease progression, especially in sites with active disease (Dzink *et al.*, 1988; Mayrand and Holt, 1988; Okuda and Takazoe, 1988; Slots and Genco, 1984; Tanner *et al.*, 1979). The extreme pathogenic potential of the black-pigmented bacteria like *P. gingivalis* has been well established (Haffajee and Socransky, 1994; Moore and Moore, 1994), especially in regard to the association of *P. gingivalis* with advancing periodontal lesions demonstrating a rapidly progressing osseous destruction (Haffajee and Socransky, 1994; Spiegel *et al.*, 1979; Tanner *et al.*, 1979). In sites with severe inflammation, *P. gingivalis* comprised a major portion of the subgingival microbiota in these studies. White and Mayrand, (1981) showed that the numbers of Gram-negative anaerobic rods were significantly higher in sites with severe inflammation than in sites with "normal" sulci, and *P. gingivalis* accounted for the majority of Gram-negative anaerobic rods in these sites in a study of 40 human periodontitis patients. *P. gingivalis* was recovered less often in sites with mild or moderate gingival inflammation and was absent in healthy sulci. These studies further underscore the association of *P. gingivalis* in the pathogenesis of adult periodontitis.

b. *Porphyromonas gingivalis* Virulence Factors

P. gingivalis possesses a unique set of properties which allow it to: 1) colonize the gingival sulcus, 2) replicate and provide for its metabolic needs, and 3) to defend itself from host defense mechanisms. These properties, known as virulence factors, help determine the pathogenicity of the organism and ultimately may contribute directly or indirectly to periodontitis-related host-tissue damage. However it must be kept in mind that virulence is not uniform across all strains of *P. gingivalis* (Neiders *et al.*, 1989).

Of the virulence factors associated with *P. gingivalis*, the proteases produced by this organism are of particular interest. *P. gingivalis* is considerably more proteolytic than other black-pigmented *Bacteroides* species (Slots and Genco, 1984). Since *P. gingivalis* is asaccharolytic and derives its nutritional needs from the breakdown of proteinaceous substrates, proteases could be involved in satisfying the virulence criterion of allowing for replication and satisfying the microorganism's metabolic needs (Carlsson *et al.*, 1984). In this regard, *P. gingivalis* proteases have been identified which degrade basement membrane and extracellular matrix molecules. These proteases include collagenase, gelatinase, phospholipase A, fibronectinase, chondroitin sulfatase and hyaluronidase (Holt and Bramanti, 1991). Such proteolytic potential would also allow for direct host tissue damage and for possible bacterial invasion of tissues.

P. gingivalis proteases have also shown the ability to degrade proteins involved in host defenses against putative periodontopathogens such as *P. gingivalis*. *P. gingivalis* proteases cleave all four IgG subclasses, IgA2, IgM, IgD and IgE in crevicular fluid and saliva as well as components of the complement cascade (Gregory *et al*., 1992; Grenier *et al*., 1989; Grenier, 1992; Nilsson *et al*., 1985). In addition, Carlsson *et al*., (1984) showed that *P. gingivalis* proteases inactivate and degrade the serum protease inhibitor molecules α1-antitrypsin and α2-macroglobulin. Armed with these proteases, *P. gingivalis* not only has the potential to promote and sustain its emergence in the periodontal pocket, but also to evade strategic host defenses and to ultimately contribute to host tissue damage.

4. Immunological Characteristics

Studies of serum antibodies in patients with various forms of periodontitis show a correlation between predominant microorganisms found in periodontal lesions and serum antibody titers to these microorganisms (Mouton *et al*., 1981; Tolo *et al*., 1981). There is also evidence that the systemic antibody response is a reflection of the host response to an infection that is associated with an episode of disease activity. Ebersole *et al*., (1987) showed that 80% of the samples from periodontally active sites in 13 patients contained detectable levels of the microorganisms to which the individual exhibited an elevated antibody response. In contrast, only 20% of the inactive sites sampled at the same time showed similar colonization.

P. gingivalis is found in the subgingival microbiota of patients with severe adult periodontitis and antibodies specific to *P. gingivalis* are found in high titers in these patients (Ebersole *et al.*, 1986; Tolo *et al.*, 1981). Ebersole *et al.*, (1986) found a significantly elevated antibody response consisting primarily of the IgG isotype specific for *P. gingivalis* in adult periodontitis patients. Mouton *et al.*, (1981) also found that *P. gingivalis* is rarely found in children and antibodies to *P. gingivalis* are low in children. The same study noted that *P. gingivalis* is rarely found in localized juvenile periodontitis or in necrotizing ulcerative periodontitis lesions and antibody titers to *P. gingivalis* in these patients are also low. However it should be noted that not all patients with adult periodontitis have elevated serum antibody titers to *P. gingivalis*.

There is still insufficient evidence to determine if the net effect of these serum antibodies to *P. gingivalis* in periodontitis is actually beneficial or detrimental to the host, but support for a protective role in severe forms of human periodontitis exists (Chen *et al.*, 1991; Gunsolley *et al.*, 1987; Williams *et al.*, 1985). A study by Cutler *et al.*, (1991) provided additional support for a protective role by showning that human serum from adult periodontitis patients contains specific IgG antibody that is opsonic for *P. gingivalis*.

B. Nonhuman Primate Model of Periodontal Disease

Human cross-sectional studies of periodontal disease obviously do not lend themselves to a longitudinal assessment of periodontitis progression. Yet it is through human cross-sectional studies that researchers have attempted and been unable to find the elusive cause and effect

relationship between specific periodontopathogens and adult periodontitis (Slots, 1979). However, ethical considerations severely hamper the complexity of longitudinal experimentation allowed in the human model and necessitate the use of suitable animal models to further our understanding in this area.

Murine, rat and hamster models, although shown to be useful in the development of *P. gingivalis* related antibody reactions with osseous (Nagahata *et al.*, 1982; Wyss and Guggenheim, 1984) and skin and peritoneal lesions (Chen *et al.*, 1987; Chen *et al.*, 1990; Chen *et al.*, 1991; Neiders *et al.*, 1989), nevertheless are limited by size constraints as well as the difficulty involved in relating their dentitions and basis for periodontal disease progression to that noted in humans.

The nonhuman primate (Nhp) provides a model that appears more appropriate for the longitudinal study of host immune responses and bacterial emergence in periodontal lesions. In particular, *Macaca fascicularis* (the cynomolgus monkey) has demonstrated a course of experimentally-induced gingivitis and periodontitis that is very similar to the clinical, histological, microbiological and immunological pattern of disease seen in humans. These Nhp originate in Southeast Asia and the females of the species (more docile than males and more desirable for use in studies) weigh from 2.5-5.7 Kg (Schou *et al.*, 1993). Their dental formula is identical to that in humans, although the teeth are overall somewhat smaller in size, the premolars all tend to be multi-rooted, large diastemata are associated with the canines, the incisors tend to meet in an edge-to edge relationship, and generalized, pronounced attrition is usually present (Auskaps and Shaw, 1957; Wirthlin *et al.*, 1992).

1. Clinical Characteristics

Naturally occurring gingivitis in *M. fascicularis* is the norm and has been found to have a similar presentation as chronic human gingivitis. The presentation in the Nhp is usually one of a mild chronic gingivitis of the papillary and marginal gingiva associated with sparse amounts of bacterial plaque and little calculus in posterior areas, and with more tooth-accumulated debris and a moderate gingivitis on the labial aspect of the incisors (Friskopp and Blomlöf, 1988; Wirthlin *et al.*, 1992). Visual signs of inflammation are present and the gingiva bleed upon gentle probing. The radiographic alveolar bone crest uniformly approaches and follows the cementoenamel junction. Rarely is a pocket deeper than 2.5 mm reported (Friskopp and Blomlöf, 1988). Although the susceptibility to gingivitis appears to be uniform among cynomolgus monkeys, the susceptibility to naturally occurring periodontitis varies greatly. Friskopp and Blomlöf, (1988) in a study of 97 cynomolgus monkeys noted that 8% had a naturally occurring periodontitis which accounted for 1% of all teeth examined.

Kornman *et al.*, (1981) studied the longitudinal progression of experimentally-induced periodontitis initiated by ligature placement. By 8-12 weeks, this experimentally generated lesion was consistent clinically, histologically and radiographically with adult periodontitis in humans. The progression of this experimentally-induced disease was divided into four clinical stages which were correlated with changes in the pocket microbiota. Stage I was a naturally occurring gingivitis, Stage II (at 3 weeks post-ligation) demonstrated gingiva with pseudopocketing and 100% bleeding on probing, Stage III (4-7 weeks post-ligation)

demonstrated severe gingival inflammation with increasing pocket depths and radiographic bone loss. The final phase, Stage IV (8-17 weeks post-ligation) demonstrated increased pocket depths, but no further increase in radiographic bone loss. It appeared from this study that experimental periodontitis progression in this model is self-limiting in its destruction. Other studies have corroborated the clinical aspects of ligature-induced periodontitis progression in *M. fascicularis* which is summarized by increased plaque accumulation, increased gingival inflammation, increased pocket depth and increased radiographic alveolar bone loss (Breckx *et al.*, 1985; Kiel *et al.*, 1983). Although Wirthlin *et al.*, (1992) demonstrated that ligature placement caused significant periodontal attachment loss in this Nhp model by two weeks post-ligation, clinical measurements of attachment levels have not been reliable markers of disease progression in this model due to small increases in pocket depths with disease, the size of the teeth, and the inherent limitations in clinical probing (Schou *et al.*, 1993).

Histological and morphological studies at the light and electron microscopic levels on ligature-induced periodontal disease in the cynomolgus monkey were reported by Breckx *et al.*, (1985, 1986), Johnson and Hopps, (1975) and Nalbandian *et al.*, (1985). Histologically, there are no significant differences between naturally-occurring gingivitis and experimental gingivitis in these Nhp, with both resembling the established lesion in humans. The size of the inflammatory infiltrate in the connective tissue predictably follows plaque accumulation, with the predominant cell type constituting plasma cells with polymorphonuclear leukocytes seen predominantly in the pocket walling off the plaque from the periodontal tissues. The

junctional epithelium is converted to a pocket epithelium; however, no evidence of attachment loss is seen.

The histological description of continued disease progression to periodontitis in this model parallels the human description. The connective tissue infiltrate of leukocytes becomes denser with a preponderance of plasma cells. Polymorphonuclear leukocytes are seen primarily associated with the plaque and bacteria in and around the ligature. Kornman *et al.*, (1981) demonstrated 4-7 week specimens that showed destruction of the connective tissue attachment to the tooth and apical migration of the junctional epithelium. Bone loss at the alveolar crest was evident. The lesion had all the signs and elements ascribed to the advanced lesion of periodontitis in humans. These histological and morphological changes described so closely parallel that seen in human periodontal disease as described by Page and Schroeder, (1982), that use of the cynomolgus monkey as a model in which to study human periodontitis has strong support.

Certain factors impact experimental disease progression in cynomolgus monkeys such as their age and the accumulated local factors already present on their teeth. Adolescent or young adult primates have been reported by Kornman, (1982) to be more resistant to induction of experimental periodontitis. Ebersole (personal communication) has indicated that Nhp with existing plaque, calculus and gingivitis present allow a faster and more predictable induction of periodontitis with ligatures than animals that have received dental prophylaxis prior to initiating disease. Manti *et al.*, (1984) found from their studies that mature female cynomolgus monkeys with an existing naturally occurring gingivitis and

erupted 3rd molars (indicating adulthood) were preferred for ligature-induced periodontitis studies.

2. Microbiological Characteristics

The bacteria comprising the dental plaque in cynomolgus monkeys closely resembles that seen in human plaque with some exceptions. Mashimo *et al.*, (1979) induced experimental gingivitis via soft diet in cynomolgus monkeys and examined the supra and subgingival plaque retrieved with mylar strips and smears and found a close similarity in composition of bacterial species compared to human plaque. Schou *et al.*, (1993) found that *M. fascicularis* has a dental plaque containing greater proportions of *Actinobacillus actinomycetemcomitans* and decreased proportions of *Actinomyces* sp. compared to human dental plaque. The significance of these differences is presently unknown.

Kornman *et al.*, (1981) found that the changes in the pocket microbiota of *M. fascicularis* during experimental gingivitis and periodontitis correlated with the clinical presentation, and also paralleled that seen in human disease rather well. Gingivitis demonstrated a preponderance of Gram-positive cocci and rods. At one to three weeks after ligation, the proportion of Gram-positive cocci decreased while numbers of motile bacteria and surface-translocating Gram-negative rods increased. By 4-7 weeks post-ligation, the subgingival plaque was dominated by Gram-negative anaerobic rods which constituted 62% of the cultivable microbiota. *P. gingivalis* was the dominant cultivable organism cultured from this lesion. As in human adult periodontitis, periodontal attachment loss in the Nhp

model was associated with increasing numbers of Gram-negative anaerobes, and *P. gingivalis* was a prominent finding.

The cynomolgus monkey model has also been used to determine the pathogenic potential *P. gingivalis* and has demonstrated an ability to be manipulated to select for specific pathogenic microorganisms through the use of systemic antibiotics. Holt *et al.*, (1988) placed rifampin-resistant mutant strains of *P. gingivalis* subgingivally and noted that a rapid and significant loss of alveolar bone occurred. This "burst" of bone loss demonstrated the ability of *P. gingivalis* to induce the progression of periodontitis in this Nhp model.

Using the longitudinal model of experimental periodontal disease progression in the cynomolgus monkey model then could allow a determination of the pathogenic potential of specific putative periodontopathogens in the periodontal pocket microbiota.

3. Immunological Characteristics

M. fascicularis demonstrates a specific humoral antibody response to various microorganisms associated with ligature-induced periodontal disease (Ebersole *et al.*, 1987; Ebersole *et al.*, 1991; Giardino, 1991). This systemic antibody response coincides with the emergence of the microorganism in the subgingival plaque during the conversion from gingivitis to progressing periodontitis. The predominant antibody response to *P. gingivalis* in this model is IgG (Ebersole *et al.*, 1991; Ebersole and Kornman, 1991), but the Nhp also

present elevated levels of IgM and IgA (Ebersole *et al.*, 1987; Ebersole *et al.*, 1991; Ebersole and Kornman, 1991 ; Holt *et al.*, 1988).

C. Immunization Against Periodontal Disease Progression

The knowledge that a specific humoral immune response occurs to putative periodontopathogens allows one to entertain the idea that active immunization (*i.e.* vaccination) against a periodontopathogenic microorganism like *P. gingivalis* or multiple such microorganisms might affect the course of periodontal disease by decreasing the numbers of the target microorganism and thereby decrease associated host tissue destruction. Such an approach, if feasible, could overcome the inherent weaknesses in the traditional approach to periodontitis prevention, as well as the shortcomings in treating established periodontal destruction discussed earlier. Moreover, it would allow us a strategy to prevent periodontitis in a population who would otherwise have no access to periodontal therapy due to economic or other constraints.

Initial work done in gnotobiotic rodent models has shown that immunization with whole-cell *P. gingivalis* or with isolated *P. gingivalis* surface proteins has been effective in altering or suppressing the severity of secondary abscess formation and/or periodontal bone loss (Chen *et al.*, 1987; Chen *et al.*, 1990; Evans *et al.*, 1992; Klausen *et al.*, 1992; Schifferle *et al.*, 1993).

Active immunization studies in *M. fascicularis* using *P. gingivalis* have shown significant elevations in antibody specific to the immunogen. In a study by Giardino *et al.*, (1996), natural antibody to *P. gingivalis* prior to immunization with *P. gingivalis* 3079.03 consisted of 75% IgG1. After immunization the main antibody response was 86-98% IgG1. Active immunization

has also been shown to elicit a functional antibody that enhanced activation and killing of *P. gingivalis* by polymorphonuclear leukocytes (Anderson *et al.*, 1995), and exhibited antibody with increased avidity compared to pre-immunization status (Hedman and Tousseau, 1989).

Ebersole *et al.*, (1991) longitudinally studied the effects of immunization with *P. gingivalis* upon the progression of ligature-induced periodontitis in *M. fascicularis*. They found that immunization elicited an elevated and specific antibody response to *P. gingivalis*, primarily of the IgG isotype. The emergence of *P. gingivalis* during disease progression was also inhibited. Nevertheless, the clinical progression of disease was not suppressed as evidenced by increased inflammation and bone loss in the ligated sites in the immunized group compared to control animals. It was concluded that subgingival ecological considerations other than the mere elimination or reduction in numbers of an individual microorganism were critical to halting experimental periodontitis progression.

A similar study done by Persson *et al.*, (1994), using *P. gingivalis* to immunize *M. fascicularis* demonstrated a significant reduction in bone loss in ligated sites of immunized animals compared to controls. Nevertheless, bone loss was not halted in these sites, nor was *P. gingivalis* cleared from any sites on ligated or unligated teeth in the immunized group. The mechanism by which immunization protected against alveolar bone destruction in this study is unclear.

It may be practical to consider the outcomes of these Nhp studies, and to conclude that perhaps elimination of one or even multiple bacterial species from the periodontal lesion may not provide the key to immunization success, even if it were feasible. The complex interactions within the periodontal pocket between bacterial species as well as between the bacteria and host

immune responses create a situation unique to periodontal disease. Elimination of one pathogen may allow opportunistic pathogens to emerge and further propagate periodontal destruction. Selective sterilization of the periodontal pocket through immunization may only be short-lived as the oral cavity affords continuous potential for immediate recolonization, and the recolonizing microbiota may be similarly pathogenic or even more so than before. Preventing the emergence of pathogenic bacteria appears worthwhile as maintaining the pocket microbiota in a Gram-positive, primarily facultative state would be more conducive to periodontal health. Although both of the immunization studies attempting to inhibit periodontitis progression in *M. fascicularis* cited above hindered the emergence of *P. gingivalis* to some degree, the clinical outcomes were diametrically opposed.

Targeting virulence factors, those characteristics that enable a pathogenic microorganism to colonize the subgingival environment and to cause host tissue destruction, presents another approach to immunization and one that aims directly at eliminating those factors directly responsible for a microorganism's pathogenic activities, without necessarily eliminating it entirely and disrupting the overall balance of the subgingival environment. Since *P. gingivalis* proteases fulfill 2 of the 3 virulence criteria enumerated earlier, namely that of enhancing bacterial replication and metabolism and evading host defense mechanisms, these would seem to be logical targets for future immunization studies.

D. Porphypain-2

Porphypain-2 is a 120 kDa cysteine protease which has recently been purified from the cell surface of *P. gingivalis* strain W12 (Lantz *et al.*, 1993; Ciborowski *et al.*, 1994). It has been shown to cleave fibrinogen *in vitro* in a manner very similar to the human protease plasmin which occurs in a stepwise manner, yielding a large fragment with a relative molecular mass of 97 kDa and smaller fragments in the range of 45-50 kDa. These fragments resemble in size the major fragments (fragments D and E) generated from fibrinogen by the action of plasmin. Plasmin plays a major role in wound healing by lysing fibrin which causes clot dissolution. It also degrades fibrinogen, clotting factors V and VIII, extracellular matrix proteins and activates host collagenases. The activity of plasmin is under strict control and is rapidly deactivated when no longer needed. If *P. gingivalis* has a plasmin-like protease on its cell surface that is not under control and cannot be turned off when it is no longer needed, then this could help explain the ubiquitous association of *P. gingivalis* with destructive periodontal disease. With such a protease, *P. gingivalis* could degrade extracellular matrix components and enhance bacterial invasion of host tissues, inhibit repair of host tissues by interfering with clot formation or by dissolving the fibrin clot, and inactivate host defense proteins. Porphypain-2 presents itself as a worthwhile target for active immunization in an attempt to alter the course of periodontal disease progression.

E. Statement of the Problem

This study will characterize the clinical, immunological, and microbiological responses to active immunization with porphypain-2 in *M. fascicularis*. Specifically, the impact of the elicited immune response on the course of experimentally-induced gingivitis and ligature-induced periodontitis will be analyzed. This study will attempt to answer the following questions.

1. **Can active immunization with porphypain-2 elicit increased antibody levels?**

2. **Is the antibody specific?**

3. **Will active immunization suppress the emergence of *P. gingivalis*?**

4. **Can active immunization alter the progression of experimental gingivitis?**

5. **Can active immunization alter the progression of ligature-induced periodontitis?**

Two specific hypotheses will be addressed in the study. The first hypothesis is that immunological and associated microbiological responses to active immunization with porphypain-2 will result in a reduction in the progression of experimental gingivitis in *M. fascicularis* compared to controls. The second hypothesis is that immunological and associated

microbiological responses to active immunization with porphypain-2 will result in a reduction or elimination of progressive ligature-induced periodontal tissue destruction in *M. fascicularis* compared to controls.

II. MATERIALS AND METHODS

A. Monkeys

Eight adult, systemically healthy, female cynomolgus monkeys were used in this study. All Nhp displayed erupted third molars and were menstruating. Animals were identified by number tattoo and were cross-referenced with housing identification located on the cages. The commercial source of these primates is Charles River Laboratories, Port Washington, NY. They had been housed in the Laboratory Animal Research Facility at the University of Texas Health Science Center at San Antonio for approximately 5-8 years. Animals were maintained in accordance with established university guidelines which are endorsed by the American Association of Laboratory Animal Care.

B. Sampling Methods and Sample Preparation

Clinical, immunological and microbiological sampling was done by one examiner (A.J.M.) at pre-determined intervals throughout the study and included clinical disease indices, blood samples, and subgingival plaque samples. Standardized longitudinal radiographs were taken at specific time points throughout the study by a second examiner (D. Cappelli).

1. Clinical Measurements

a. Clinical Parameters of Disease

Plaque scores for experimental and control teeth were determined using the plaque index of Silness and Löe, (1964). The plaque index and all other clinical parameters were analyzed at the mesial-facial, mid-facial, distal-facial and mid-lingual surfaces of all study teeth.

A modified bleeding index using a zero-to-5 point scale was used in order to provide an extended range of responses for this parameter (Appendix A). Presence or absence of bleeding was annotated at a site 20 seconds after probing was done.

Probing depths were determined using the same manual Michigan O Probe with Williams Markings (Hu-Friedy Co.) throughout the study. All recordings were made to the nearest millimeter and uniformly rounded upward where necessary.

Clinical attachment level calculations were made by adding recession level recordings in millimeters from the cemento-enamel junction, to the probing depth recordings.

Reproducibility studies were done to determine the variance of repeated probing depth and clinical attachment level measurements made on the same animals by the same examiner. Two full mouth exams in 4 monkeys were performed 15 minutes apart using the same manual periodontal probe. 500 total sites representing 125 teeth were examined. For repeated probing depth measurements, 96.8% of sites were in total agreement, and 100% of sites were exactly the same or within 1 mm.

For attachment level measurements, 95.0% of measurements were identical, and 98.8% of recordings were the same or within 1 mm. No measurements exceeded $\pm$ 2 mm.

b. Standardized Longitudinal Radiographs

Additional clinical data was gathered for Computer Assisted Densitometric Image Analysis (CADIA) (Brägger *et al.*, 1988) in the form of numerically-encoded, standardized longitudinal radiographs. Radiographs were obtained using custom made acrylic stents which allowed uniform reproduction of radiographic image geometry.

2. Immunological Sampling

Serum was prepared from venipuncture blood samples gathered from the femoral vein of each Nhp that were allowed to clot for 1 hour at room temperature. Samples were then refrigerated at 4° C overnight. On the second day, samples were centrifuged at 4° C at 3,000 rpm for 30 minutes, and the serum removed. The serum samples were then stored at -20° C until used for ELISA studies.

3. Microbiological sampling

Subgingival plaque samples were procured from the mesial-facial proximal areas of all experimental and control teeth by first gently wiping away all supragingival plaque with cotton gauze and then using a sterile curette. Samples were pooled for each given experimental or control sextant and placed into sequentially numbered Eppendorf tubes which held 100 μl of Tris-EDTA buffer. 100 μl of 0.5 M NaOH was then added to each tube and each sample sonicated to lyse the bacteria. Samples were stored at -20° C until used for DNA analysis.

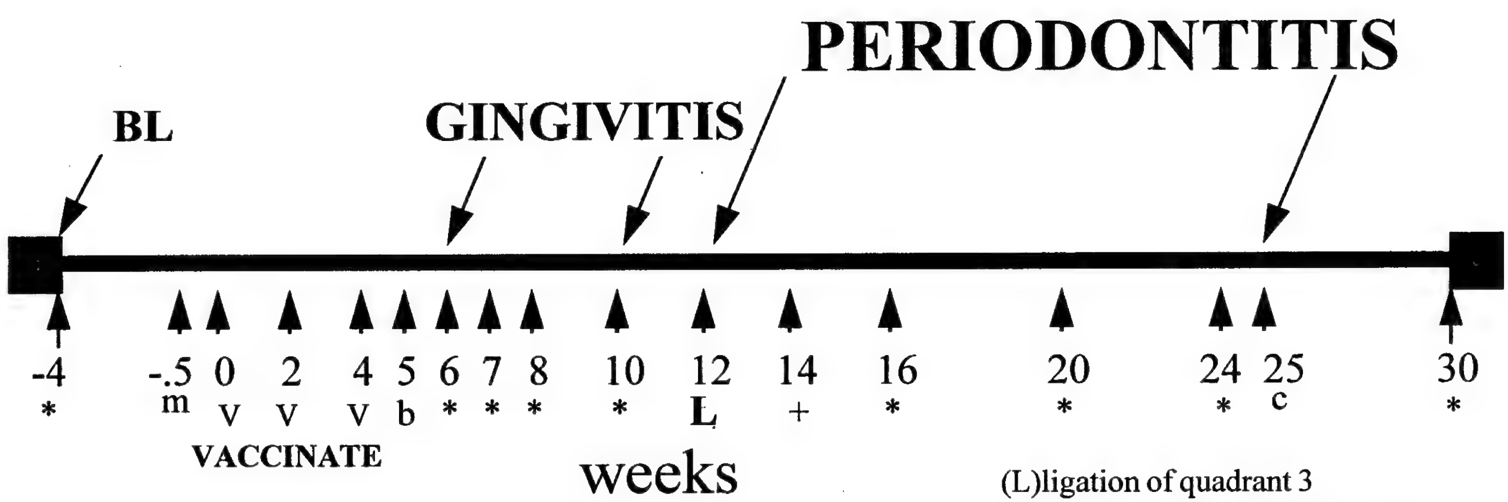
C. Experimental Protocol

Baseline samples were procured at -4 weeks. Figure 1 outlines the protocol and all sampling performed. ELISA was initially performed on baseline serum samples to determine anti-*P. gingivalis* IgG levels. Using these values along with baseline attachment levels and bleeding scores, the Nhp were divided into 2 balanced groups of 4 animals each which were statistically similar (Table 1).

At time 0, Group 1 Nhp were immunized subcutaneously with 100 μg of porphypain-2 antigen preparation in Incomplete Freund's Adjuvant (IFA), while Group 2 Nhp were injected with a placebo preparation in IFA (APPENDIX B). Booster immunizations were given at weeks 2 and 4 using the same amounts. Immunization was done by a person (S. Cox) other than the main study examiner, the latter remaining blinded as to the identity of the immunized group throughout the duration of the study.

Figure 1: Study Protocol. This timeline represents the experimental protocol involving the two groups of Nhp with immunizations or sham injections and sampling taking place as outlined.

Study Protocol



(L)ligation of quadrant 3
(*)micro, clinicals, blood, x-rays
(+)micro, blood only
(m)micro only
(b)blood only
(c)clinicals only

Table 1

NHP STUDY GROUPS

Immunized Group

Animal #	ELISA Units (IgG to *P. gingivalis*)	Attachment level (mm)	Bleeding Index
N11	5.1	0.3	0.13
R01	111.8	1.0	0.83
R03	29.7	1.4	1.67
S27	28.2	0.9	0.42
Group Means	43.7 ± 46.8	0.9 ± 0.3	0.76 ± 0.67

Non-immunized Group

Animal #	ELISA Units (IgG to *P. gingivalis*)	Attachment level (mm)	Bleeding Index
P95	40.9	0.6	1.58
R07	10.0	1.2	0.83
S25	74.0	1.0	0.38
S33	31.6	1.0	0.79
Group Means	39.1 ± 26.6	0.9 ± 0.3	0.92 ± 0.49

Blood was drawn at week 5, and an ELISA performed to determine adequate antibody level response to the immunization prior to proceeding with the gingivitis phase of the study. An antibody response of 50 times baseline levels was set as a goal, however a minimum antibody response of 5 times baseline levels was considered necessary for continuation with the clinical portion of the study. Adequate antibody levels were attained, and the gingivitis phase was initiated at week 6 by switching the animals to a soft diet and without providing any mechanical oral hygiene. The soft diet was maintained throughout the gingivitis phase ending at week 10, and was continued through the end of the periodontitis phase at week 25.

Experimental teeth (3-5, 3-6, 3-7) were ligated with 3-0 silk sutures at week 12 in both the immunized and non-immunized Nhp groups. (These teeth will also be referred to as "ligated sites" denoting those sites that ultimately received ligatures during the periodontitis phase of the study.) The contralateral teeth (4-5, 4-6, 4-7) in each animal served as non-ligated intra-animal controls. The ligature-induced periodontitis phase persisted for 13 weeks with ligature removal at week 25. Additional sampling was done 5 weeks after ligature removal to determine any changes in measured parameters subsequent to ligature removal.

D. ELISA

1. Procedure and Analysis

An Enzyme Linked Immunosorbent Assay (ELISA) was used to determine the level, specific isotype, and kinetics of the antibody response to active immunization with porphypain-2. Specifically, whole formalinized bacteria (*P. gingivalis* 3079.03) were

diluted in $NaC0_3$ buffer at pH 9.6 to obtain a concentration that would give a known optical density (OD) reading when analyzed at 405 nm on a spectrophotometer. 96-well polystyrene microtiter plates (Titertek®) were coated with these bacteria, incubated for 4 hours at 37° C and stored at 4° C until used.

For all antibody determinations, various dilutions of monkey sera in phosphate buffered saline (PBS) and 0.05% Tween 20 (Sigma Chemical Co.) were incubated for 2 hours at room temperature on a rotator. Dilution ranges used are presented in Table 2. Sera were diluted so that the OD readings fell within the linear range of the standard curve developed on each plate. All dilutions were assayed in triplicate.

After three 5 minute washings with 0.9% NaCl containing 0.05% Tween 20, incubation with affinity purified goat anti-human IgG, IgM or IgA antisera (Calbiochem IgG #902225, IgM #902571, and IgA #902664) at 1:500 concentration in 1% Blotto (Ebersole, personal communication) for 2 hours at room temperature took place.

Three additional washings as above were performed, and incubation with affinity purified rabbit anti-goat IgG conjugated to alkaline phosphatase (Sigma #083H-8850) at 1:1,000 concentration took place overnight at room temperature.

The following day, washing as described above was performed and p-nitrophenylphosphate (1 mg/ml in 0.05M $NaC0_3$, pH 9.8 with 1mM $MgCl_2$; Sigma 104 Phosphate Substrate) was added as the substrate. The resultant colorimetric reactions were terminated by the addition of 1N NaOH.

A serially-diluted serum standard was included in duplicate on each plate along with background control wells. The reference standard sera were obtained from nonhuman

Table 2

SERUM DILUTIONS FOR *P. GINGIVALIS* 3079.03 COATED ELISA PLATES

	IgG	IgM	IgA
Baseline sera	1:100	1:25	1:50
Immunized sera	1:400 to 1:1600	1:100 to 1:400	1:100 to 1:400
Reference standard sera	1:400 (100 EU)	1:50 (12.5 EU)	1:40 (10 EU)

primates hyperimmunized with *P.gingivalis* 3079.03 (Ebersole *et al.*, 1991). The resultant reaction of all sera compared to the standard was determined spectrophotometrically at 405 nm (Biomek). Linear regression analysis related the OD of the experimental sera to the logarithm of the antibody activity in the standard and was used to derive ELISA values for antibody titers present.

2. ELISA for Anti-Porphypain-2 IgG

Similarly, to determine the specific IgG response to porphypain-2, additional microtiter plates were coated with 2.0 μg/ml of porphypain-2 antigen preparation and processed as outlined above with the exception that the plates were blocked with 1% Blotto for two hours prior to beginning the ELISA. Serum dilutions ranged from 1:200 to 1:50,000. Standard sera and polyclonal reagents were diluted as before.

3. ELISA Specificity Determination

In order to monitor the specificity of the ELISA, baseline and week 5 (immediate post-immunization) sera were analyzed to determine antibody responses to *Actinobacillus actinomycetemcomitans* 6250.6 and *Prevotella intermedia* 6235.2. Serum and standard dilutions used are noted in Table 3. Goat anti-human reagents were used at 1:1,000 concentration, otherwise the procedures were followed as already outlined.

Table 3

SERUM DILUTIONS FOR *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* 6250.6 AND *PREVOTELLA INTERMEDIA* 6235.2 COATED ELISA PLATES

***A. actinomycetemcomitans* 6250.6**

	IgG	IgM	IgA
Baseline sera	1:1600	1:1600	1:200
Immunized sera	1:1600	1:1600	1:200
Reference standard sera	1:800 (100 EU)	1:400 (50 EU)	1:100 (12.5 EU)

***P. intermedia* 6235.2**

	IgG	IgM	IgA
Baseline sera	1:200	1:100	1:400
Immunized sera	1:200	1:200	1:400
Reference standard sera	1:800 (100 EU)	1:400 (50 EU)	1:400 (12.5 EU)

E. Checkerboard DNA Probe Analysis

A checkerboard DNA probe technique utilizing a "minislot" and "miniblotter"apparatus as described by Socransky *et al.*, (1994) was used to analyzed pooled plaque samples for the presence of 16 different Gram-positive and Gram-negative bacterial species (Table 4).

Number coded, solublized plaque samples were boiled for 5 minutes, cooled on ice for 5 minutes, and neutralized with 800μl of 5M ammmonium acetate. Following vortexing, samples were pipetted into the horizontal channels of the "minislot" apparatus which was aligned over a positively charged nylon membrane (Boehringer Mannheim Co.). In order to minimize the effect of processing variability between probes, solublized plaque samples from two different sampling sessions were included on each membrane. DNA present in the samples was linked to the membrane with a 30 second ultraviolet exposure (Stratolinker®, Stratagene Co.). After baking for 20 minutes at 120°C, the membrane was pre-hybridized at 42°C for 1 hour in a solution including 25.0 ml formamide, 5.0 ml Denhardt's solution, 3.0 ml herring sperm DNA, 5.0 ml stock block, and 12.5 ml 25 mM sodium phosphate, pH 6.5. Simultaneously, digoxigenin-labeled whole genomic DNA probes (supplied by Dr. S. Socransky, Forsyth Dental Center, Boston MA) were combined with hybridization buffer (10.0 ml formamide, 0.4 ml Denhardt's solution, 0.4 ml herring sperm DNA, 2.0 ml stock block, 2.0 mg dextran sulfate, 20 mM sodium phosphate, pH 6.5), boiled and cooled on ice, and 145 μl of each probe pipetted into the lanes of the "miniblotter" apparatus layed over the prehybridized membrane which had been rotated 90^0 from its original orientation so that the probe lanes would intersect the plaque sample lanes. The membrane was then hybridized overnight at 42°C suspended over a shaking water bath.

Table 4

DNA PROBES UTILIZED FOR "CHECKERBOARD" DNA HYBRIDIZATION

DNA Probe	Volume per lane (μl)
381 *Porphyromonas gingivalis*	4
Y4 *Actinobacillus actinomycetemcomitans*	3
364 *Fusobacterium nucleatum*	6
371 *Campylobacter rectus*	4
28285 *Bacteroides fragilis*	6
T14 *Actinomyces viscosus*	12
SS1 *Streptococcus sanguis*	12
27335 *Streptococcus intermedius*	5
B1 *Treponema denticola*	3
8944 *Prevotella nigrescens*	5
51146 *Campylobacter showae*	3
10558 *Streptococcus gordonii*	6
13541 *Selenomonas noxia*	3
23834 *Eikenella corrodens*	3
1084 *Campylobacter gracilis*	3
581 *Prevotella intermedia*	3

The following morning, the membrane was washed with 20 mM sodium phosphate and 1% SDS at 65^0 C in a Disk Wisk apparatus (Schleicher & Scheull Co.), followed by washings with maleic acid buffer, pH 8.0, and 1% casein in 100 mM maleic acid, pH 7.5 on a rotator. The membrane was then incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim Co.) for 30 minutes at room temperature on a rotator, and again washed in succession with maleic acid buffer, pH 8.0, and 0.05 M $MgCl_2$ buffer, pH 9.5 on a rotator. Lumi-Phos™ 530 (Boehringer Mannheim Co.) was spread over the membrane which was then wrapped in plastic wrap and incubated at 37^0 C for one hour. The DNA probe was then developed by exposing the membrane to radiographic film for 3 to 4.5 hours.

To quantitate the relative amounts of bacterial DNA present in each plaque sample and thereby determine approximate numbers of each bacterial species present in the samples, a computerized, optically enhanced densitometry system (pdi QS30, Fisher Scientific Co.) was used to analyze each developed DNA probe. The reactions produced by reference standards (1×10^6, 1×10^5, 1×10^4; supplied by Dr. S. Socransky) for each microorganism and included on each probe were utilized to develop reference curves to which all reactions on each probe were compared. The program then assigned a specific value for the relative amount of DNA present for each analyzed microorganism in each plaque sample.

F. Computer Assisted Densitometric Image Analysis

Standardized longitudinal radiographic images of experimental and control teeth were analyzed for density changes in crestal areas of interproximal bone. Using a standard subtraction

radiography technique, digitized images of radiographs were compared to their respective baseline images, and CADIA was employed to determine specific areas of gain or loss of crestal bone density and assign a value expressed in CADIA units.

Specifically, all numbered radiographic images were digitized, and each image was then aligned to its baseline image. Each digitized image consisted of 512 x 480 pixels which were converted into intensity gray levels from 0 (darkest) to 256 (lightest). A gamma correction algorithm adjusted for minor discrepancies in film contrast and angulation as images were input. Specific areas of interest (AOIs) of 8 x 8 pixels were determined in the interproximal crestal areas between 3-8/3-7, 3-7/3-6, 3-6/3-5 or 4-8/4-7, 4-7/4-6, 4-6/4-5 on each baseline image. Also a fourth AOI was placed as a control in an area of no expected change. Each subsequent radiograph was subtracted from its baseline image, and a threshold level representing the standard deviation of the distribution of gray tone levels between compared images determined using histogram analysis. CADIA values were then reported for those density changes which exceeded twice the standard deviation of the image. CADIA values for each sextant were expressed as the sum of all positive and negative density changes corrected by the value derived from the control AOI. CADIA values reported for each sextant represented the total area of change times the mean density change present.

Prior to performing CADIA, calibration of the examiner was accomplished by performing multiple repeated measures on duplicate sets of Nhp radiographs.

G. Statistical Analyses

Descriptive statistics were obtained for data from both groups using Stat-sak™ and Minitab™ statistical software (State College, PA) on an IBM PC. Point-to-point analyses, grouped point comparisons, as well as correlation studies were performed using the Student *t*-test or nonparametric analyses (Mann-Whitney U test, Krukall-Wallis ANOVA, and Spearman rank order correlation analysis) where considered appropriate. Unless otherwise indicated, *P* values of results to follow are reported using the Student *t*-test.

III. RESULTS

A. Immunological Results

1. Antibody Responses Immediately Post-Immunization

All of the Nhp in this study demonstrated baseline antibody levels to *P. gingivalis* 3079.03 and to the specificity test microorganisms *A. actinomycetemcomitans* 6250.6 and *P. intermedia* 6235.2. Table 5 provides descriptive statistics of naturally existing serum antibody levels in the Nhp groups. At the first post-immunization serum sampling at week 5, a significant increase in mean anti-*P.gingivalis* IgG antibody levels was noted which represented an approximately 11-fold increase over baseline levels in the immunized Nhp group. This level was deemed adequate to proceed to the gingivitis phase of the study. Non-immunized controls showed no changes. Figure 2 shows the baseline and immediately post-immunization antibody responses in the immunized animals for *P. gingivalis*, *A.actinomycetemcomitans*, and *P. intermedia.* There were no significant changes in levels of any antibody isotype to the latter two microorganisms, affirming the specificity of the ELISA test.

Table 5

DESCRIPTIVE STATISTICS OF NATURALLY EXISTING NHP SERUM ANTIBODY LEVELS TO *P. GINGIVALIS*, *A. ACTINOMYCETEMCOMITANS*, AND *P. INTERMEDIA*

Immunized Group

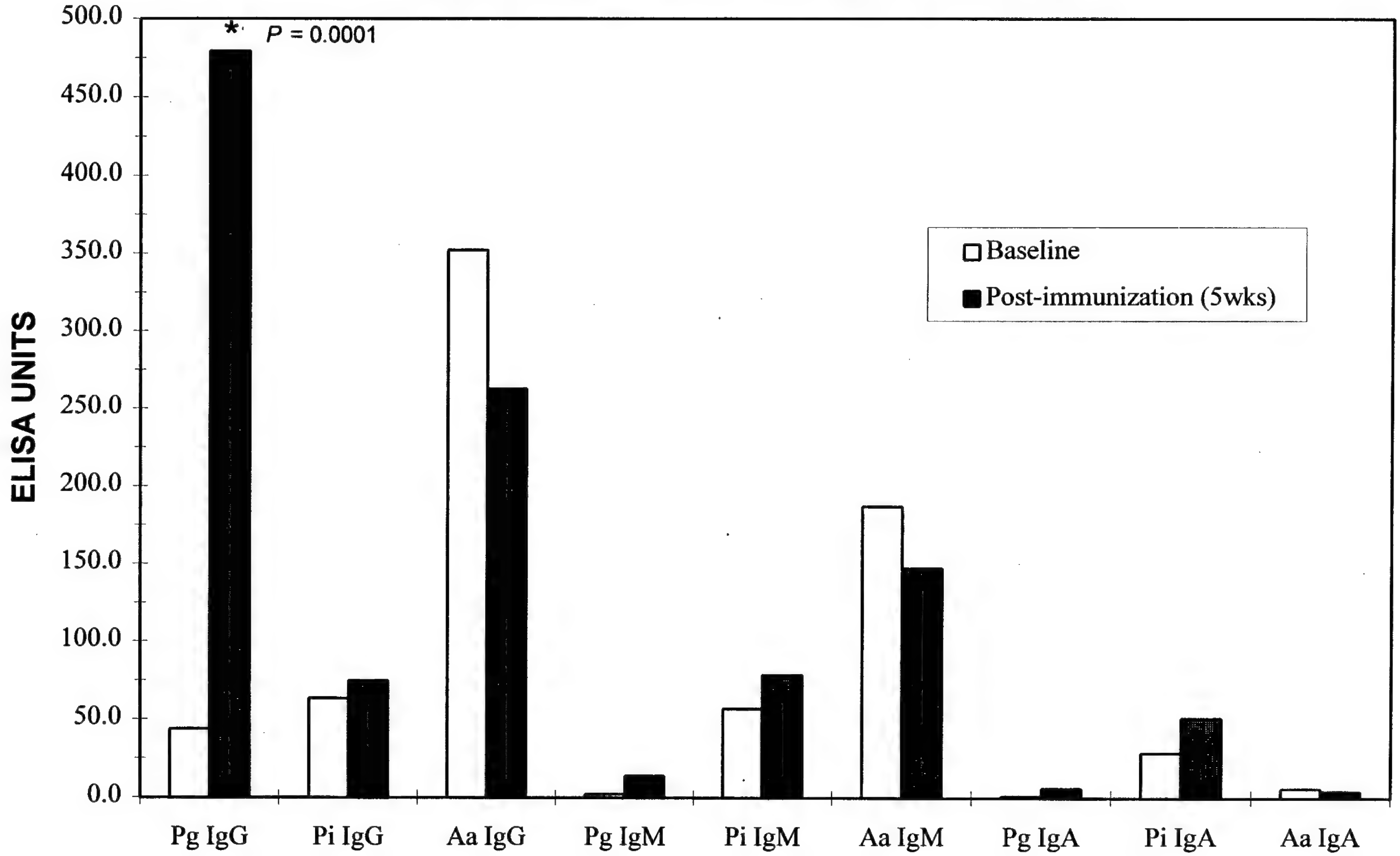
Bacterial species	Isotype	Mean	Median	SD	SEM	Min	Max
Pg	IgG	43.7	28.9	46.8	23.4	5.1	111.8
	IgM	2.0	1.75	1.6	0.8	0.5	4.1
	IgA	1.0	0.2	1.7	0.8	0.1	0.2
Aa	IgG	352.3	256.8	246.0	123.0	178.8	716.7
	IgM	186.9	136.8	119.7	59.8	110.1	363.9
	IgA	5.9	6.0	0.4	0.2	5.5	6.4
Pi	IgG	63.4	60.2	24.8	12.4	40.0	93.2
	IgM	56.9	30.6	60.9	30.4	19.5	147.2
	IgA	28.6	28.6	4.9	2.4	22.6	34.5

Non-immunized Group

Bacterial species	Isotype	Mean	Median	SD	SEM	Min	Max
Pg	IgG	39.1	36.3	26.6	13.3	10.0	74.0
	IgM	2.2	2.2	1.6	0.8	0.7	3.8
	IgA	0.7	0.6	0.5	0.3	0.1	1.4
Aa	IgG	650.9	673.9	257.4	128.7	380.1	875.8
	IgM	236.9	245.0	76.5	38.3	135.8	321.9
	IgA	13.1	13.4	3.5	1.7	8.6	16.9
Pi	IgG	89.1	104.4	32.6	16.3	40.2	107.4
	IgM	72.8	64.3	57.2	28.6	12.4	150.3
	IgA	60.4	60.1	46.9	23.5	13.2	108.3

Figure 2: Antibody Responses at Baseline and Post-immunization for *Pg*, *Pi* and *Aa*. Shown are mean baseline (week -4) and first post-immunization (week 5) IgG, IgM and IgA antibody titers in the Nhp group immunized with porphypain-2. *A. actinomycetemcomitans* and *P. intermedia* results confirmed the specificity of the ELISA. Values are expressed in ELISA units.

Antibody Responses at Baseline and Post-immunization for *Pg*, *Pi* and *Aa*



2. Antibody Isotype and Kinetic Responses

a. Natural Antibody Levels

At baseline, there were no significant differences in any of the tested antibody isotype levels between the immunized and non-immunized control groups.

The predominant antibody isotype to *P. gingivalis* in both Nhp groups was IgG. The baseline serum IgG antibody levels to *P. gingivalis* for the eight study Nhp ranged from 5.1 to 111.8 ELISA units (EU) (Figure 3). Baseline serum IgG antibody levels to the protease, porphypain-2 were also determined and ranged from 20.4 to 324.9 EU (Figure 4). A wide variability of antibody levels to whole-cell *P. gingivalis* and to the protease antigen were observed among the Nhp.

The baseline serum IgM antibody levels to *P. gingivalis* ranged from 0.5 to 4.1 EU (Figure 5), and IgA levels to *P. gingivalis* ranged from 0.1 to 3.5 EU (figure 6). A similar variability of IgM and IgA responses as observed for IgG levels was seen among the Nhp. IgM and IgA levels specific to porphypain-2 were not determined in this study.

b. Serum Antibody Responses to Active Immunization

Active immunization of the Nhp was accompanied by a substantial increase in antibody response. The predominant anti-*P. gingivalis* antibody isotype response to active immunization with porphypain-2 was IgG which is shown in Figure 7. After

Figure 3: Baseline Serum IgG Antibody Levels to *P. gingivalis*. Represented are naturally existing IgG Antibody levels at study baseline to *P. gingivalis* as expressed in ELISA units. Nhp identification numbers are shown on the y-axis. The bars represent the mean of triplicate assays of each Nhp serum sample. The immunized group consisted of animals #N11, R01, R03, and S27. Nhp #P95, R07, S25 and S33 constituted the sham-injected control group. These values along with mean baseline probing depths and bleeding scores were initially used to divide the animals into two balanced study groups which were statistically similar.

Baseline Serum IgG Antibody Levels to *P. gingivalis*

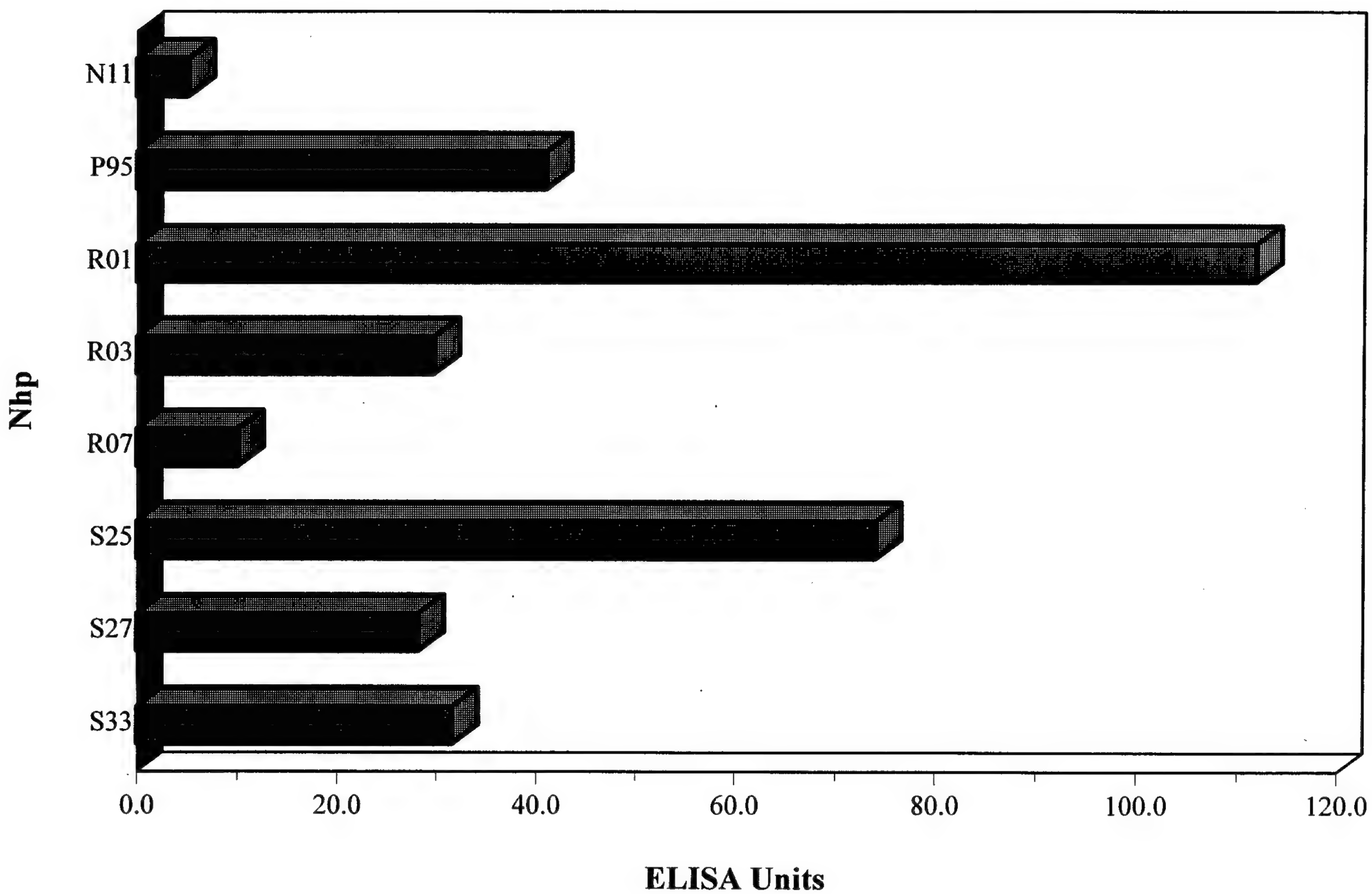


Figure 4: Baseline Serum IgG Antibody Levels to Porphypain-2. Represented are naturally existing IgG Antibody levels at study baseline to the *P. gingivalis* protease porphypain-2 as expressed in ELISA units. Nhp identification numbers are shown on the y-axis. The bars represent the mean of triplicate assays of each Nhp serum sample. The immunized group consisted of animal #N11, R01, R03, and S27. Nhp #P95, R07, S25 and S33 constituted the sham-injected control group.

Baseline Serum IgG Antibody Levels to Porphypain-2

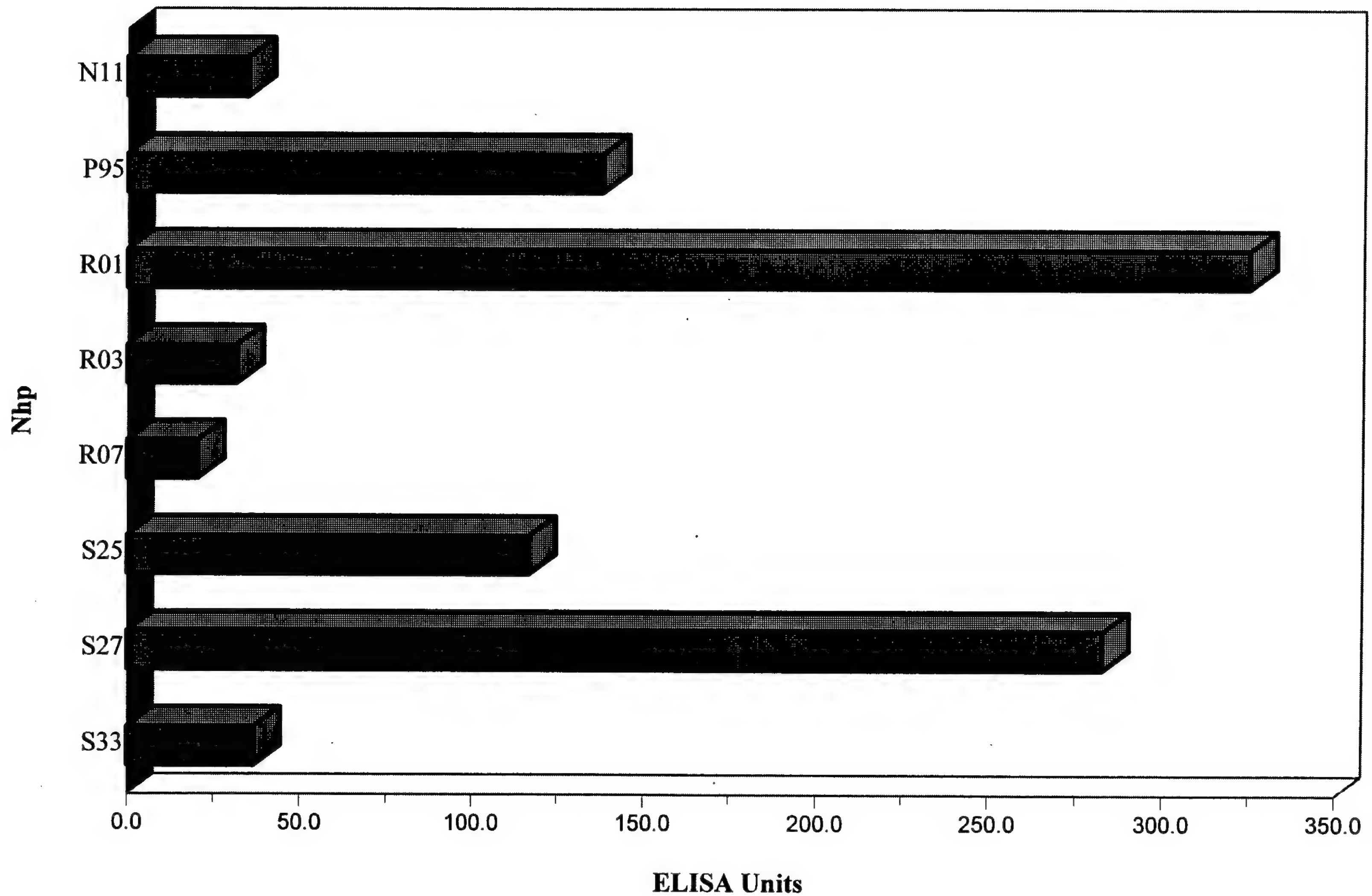


Figure 5: Baseline Serum IgM Antibody Levels to *P. gingivalis*. Represented are naturally existing IgM Antibody levels at study baseline to *P. gingivalis* as expressed in ELISA units. Nhp identification numbers are shown on the y-axis. The bars represent the mean of triplicate assays of each Nhp serum sample. The immunized group consisted of animal #N11, R01, R03, and S27. Nhp #P95, R07, S25 and S33 constituted the sham-injected control group.

Baseline Serum IgM Antibody Levels to *P. gingivalis*

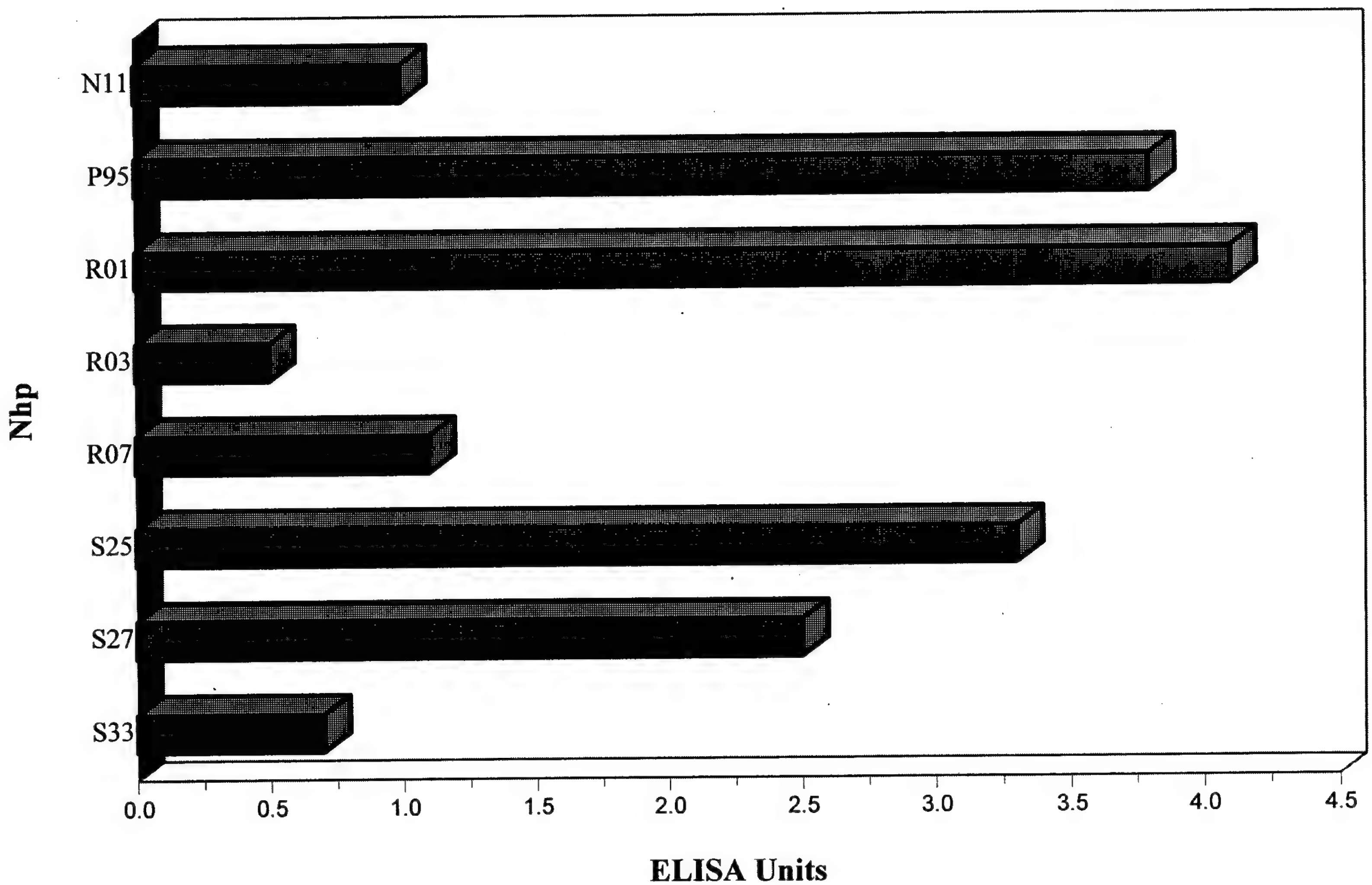


Figure 6: Baseline Serum IgA Antibody Levels to *P. gingivalis*. Represented are naturally existing IgA Antibody levels at study baseline to *P. gingivalis* as expressed in ELISA units. Nhp identification numbers are shown on the y-axis. The bars represent the mean of triplicate assays of each Nhp serum sample. The immunized group consisted of animal #N11, R01, R03, and S27. Nhp #P95, R07, S25 and S33 constituted the sham-injected control group.

Baseline Serum IgA Antibody Levels to *P. gingivalis*

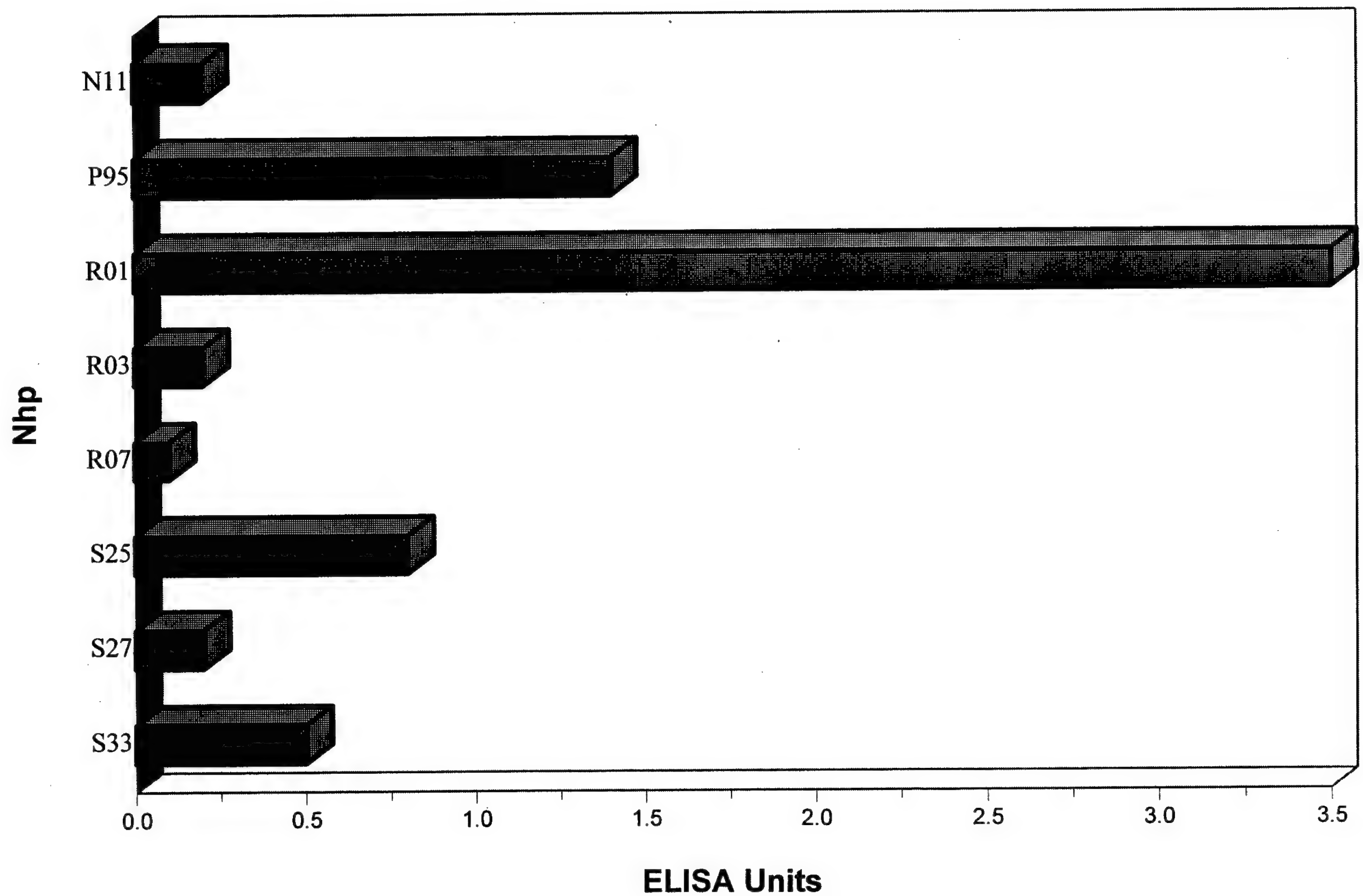
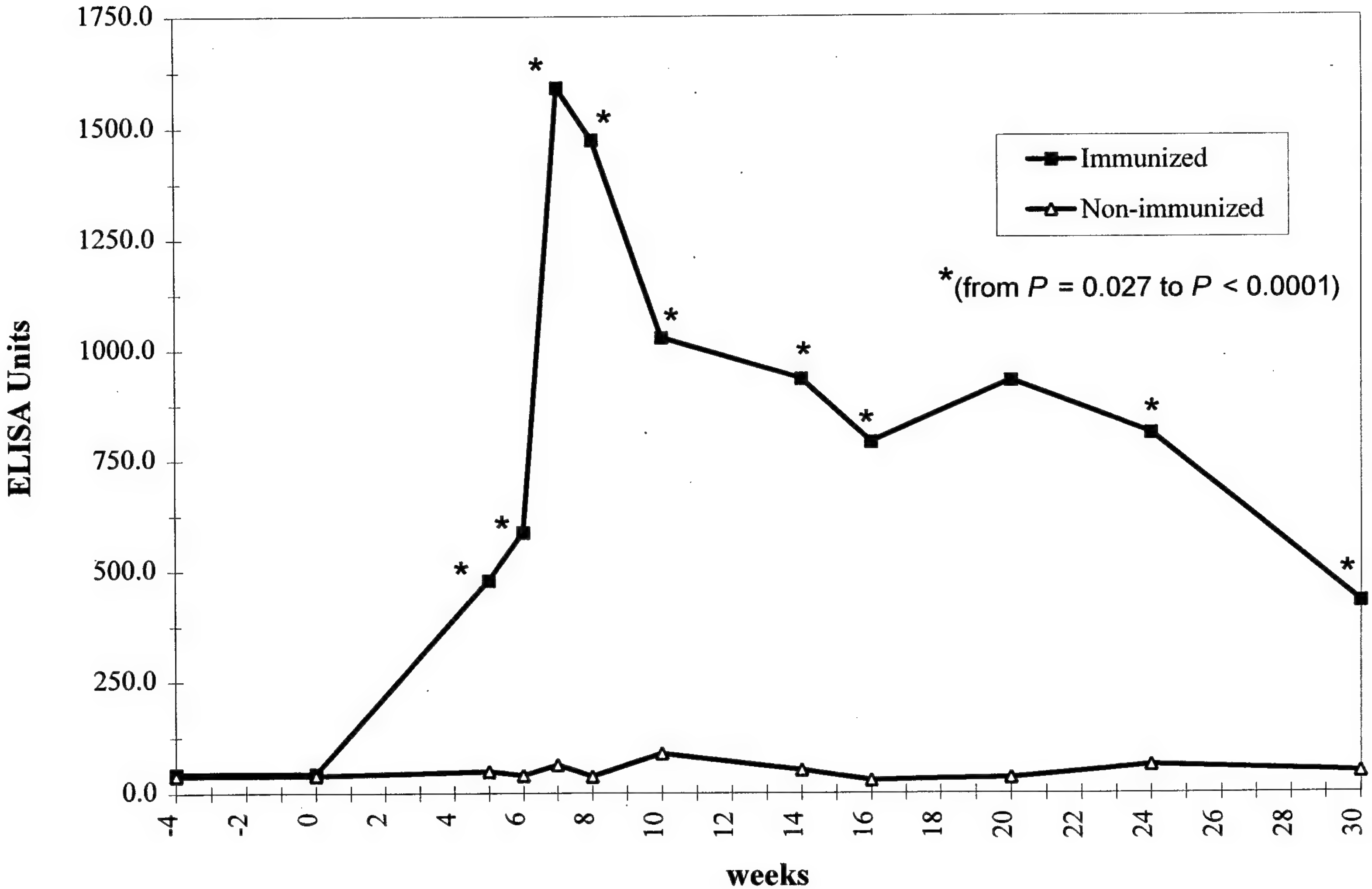


Figure 7: IgG Antibody Response to *P. gingivalis*. This chart represents the dramatic and sustained specificity and kinetics of the serum IgG antibody response to *P. gingivalis* following immunization with porphypain-2 as expressed in ELISA units. All asterisks denote significance from baseline values. Immunizations occurred at week 0, with booster immunizations at weeks 2 and 4.

IgG Antibody Response to *P. gingivalis*



immunization, there was a rapid increase in IgG levels to week 7 after which they remained significantly elevated ($P = 0.027$ to $P < 0.0001$) compared to baseline levels through the end of the study at week 30. The maximum mean IgG response reached (1591.6 EU), was 36-fold higher than baseline values. This demonstrates both the dramatic increase in antibody levels post-immunization as well as the specificity of the response. There were no significant changes in anti-*P. gingivalis* antibodies in the non-immunized Nhp. Post-immunization IgG levels in the immunized group became signficantly higher than the non-immunized group beginning at week 5 and lasted until the end of the study (from $P < 0.03$ to $P < 0.00001$).

Immunization also demonstrated an elevated and specific IgG antibody response to porphypain-2 antigen which is shown in Figure 8. This antibody response reached a maximum value at week 14 (32,680.5 EU) which represented a 194-fold increase over baseline levels. The kinetic pattern of the IgG response to the protease antigen was similar to that seen for the IgG response to whole-cell *P. gingivalis* and remained significantly elevated compared to baseline ($P < 0.02$ to $P = 0.0001$), and to the non-immunized control group ($P < 0.02$ to $P = 0.0001$) through the end of the study.

The relationship between the IgG antibody responses to *P. gingivalis* and to porphypain-2 is presented in a scatter plot diagram in Figure 9 where IgG to the protease antigen is plotted against IgG to *P. gingivalis*. A clear association is noted.

Figure 8: IgG Antibody Response to Porphyain-2. This chart represents the extremely dramatic and sustained specificity and kinetics of the serum IgG antibody response to porphypain-2 following immunization with porphypain-2 as expressed in ELISA units. All asterisks denote significance from baseline values. Immunizations occurred at week 0, with booster immunizations at weeks 2 and 4.

IgG Antibody Response to Porphypain-2

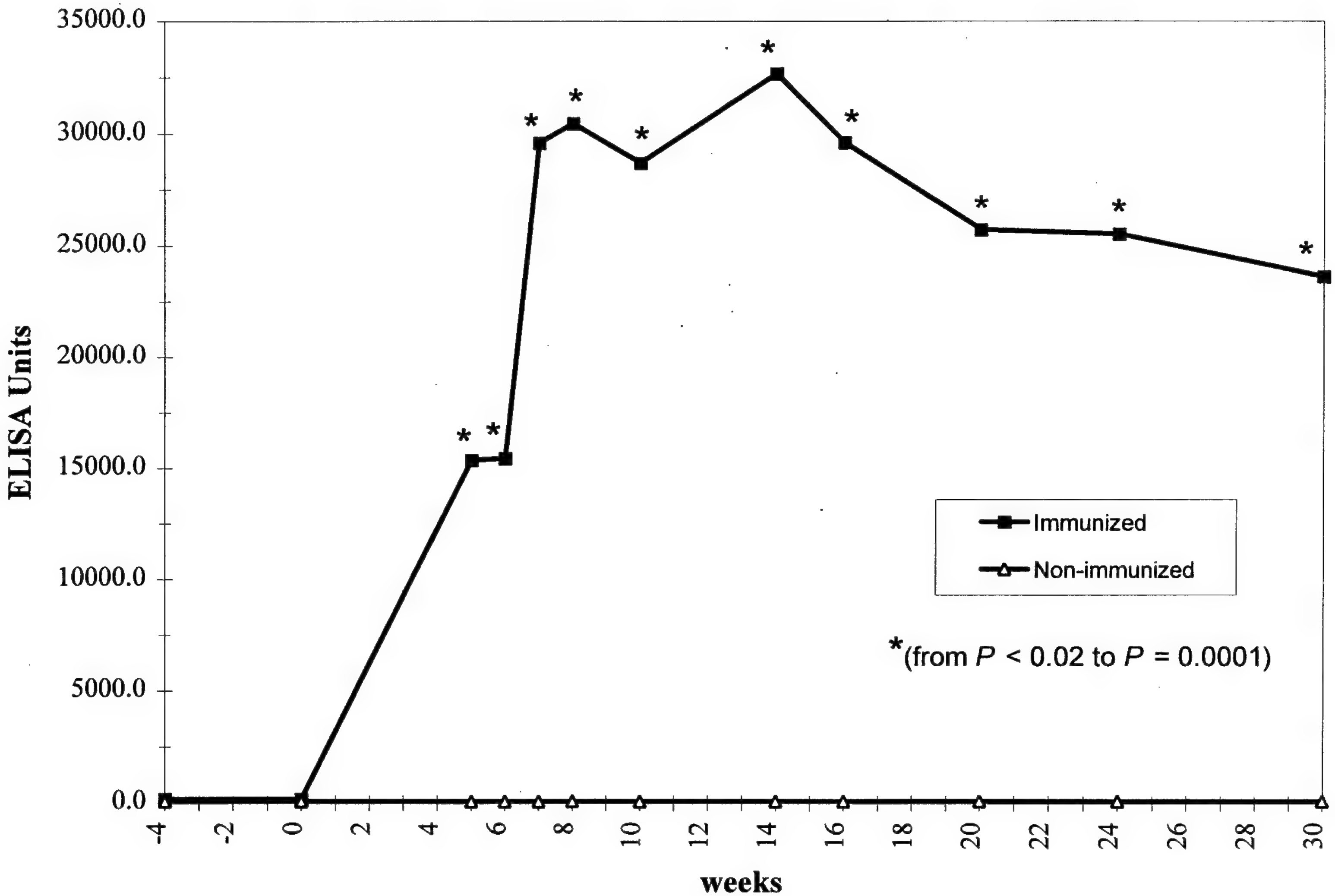
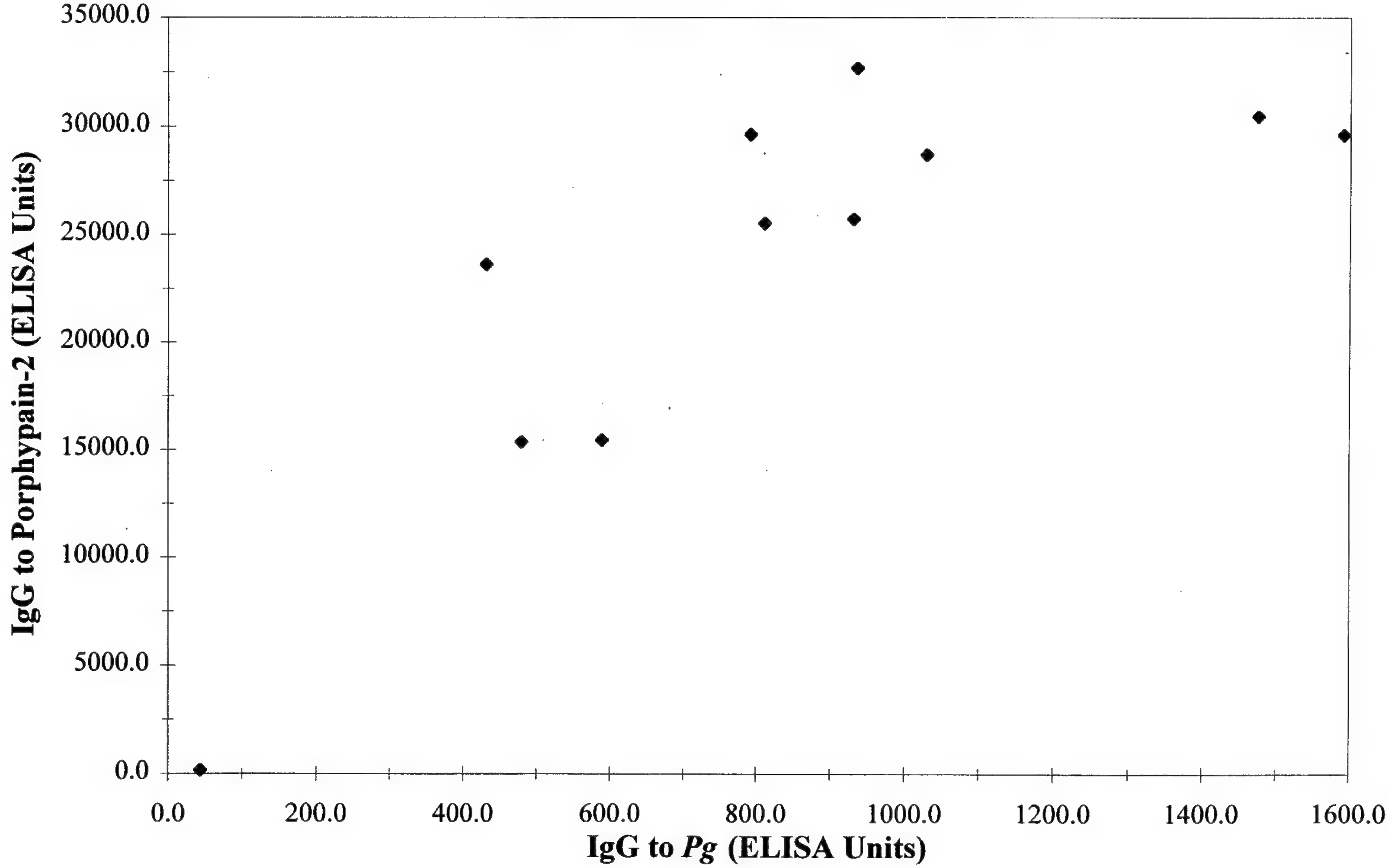


Figure 9: Scatter Plot Diagram of IgG Antibody to *P. gingivalis* versus IgG Antibody to Porphypain-2. Serum IgG to *P. gingivalis* (x-axis) is plotted against IgG to porphypain-2 (y-axis) using mean group titers expressed in ELISA units.

Scatter Plot Diagram of IgG Antibody to *P. gingivalis* versus IgG Antibody to Porphypain-2



The anti-*P. gingivalis* IgM and IgA (Figure 10) responses to immunization demonstrated non-significant increases over baseline levels which rapidly diminished to near baseline levels. There were no significant differences between the immunized and non-immunized groups.

B. Clinical Results

Overall, the results of immunization revealed no detectable adverse systemic responses in the Nhp. Along with experimental periodontal disease progression, however, significant clinical changes took place in the ligated sites in both Nhp groups.

1. Clinical Periodontal Disease Parameters

a. Plaque Scores

Mean plaque scores for all experimental and control sites are summarized in Figure 11. For all ligated and non-ligated sites in both Nhp groups, significant increases in plaque accumulation were noted beginning with the establishment of the gingivitis phase and continuing through the end of the periodontitis phase at week 25. At this point, a normal, hard diet was reinstituted, and plaque levels in all sites decreased to levels not significantly different from baseline by study end at week 30. Significant increases in plaque accumulation beyond that seen during the gingivitis phase accompanied the placement of ligatures at 16 weeks in the experimental sites

Figure10: IgM and IgA Antibody Responses to *P. gingivalis*. This chart represents the kinetics of the serum IgM and IgA antibody responses to *P. gingivalis* following immunization with porphypain-2 as expressed in ELISA units. Immunizations occurred at week 0, with booster immunizations at weeks 2 and 4.

IgM and IgA Antibody Responses to *P. gingivalis*

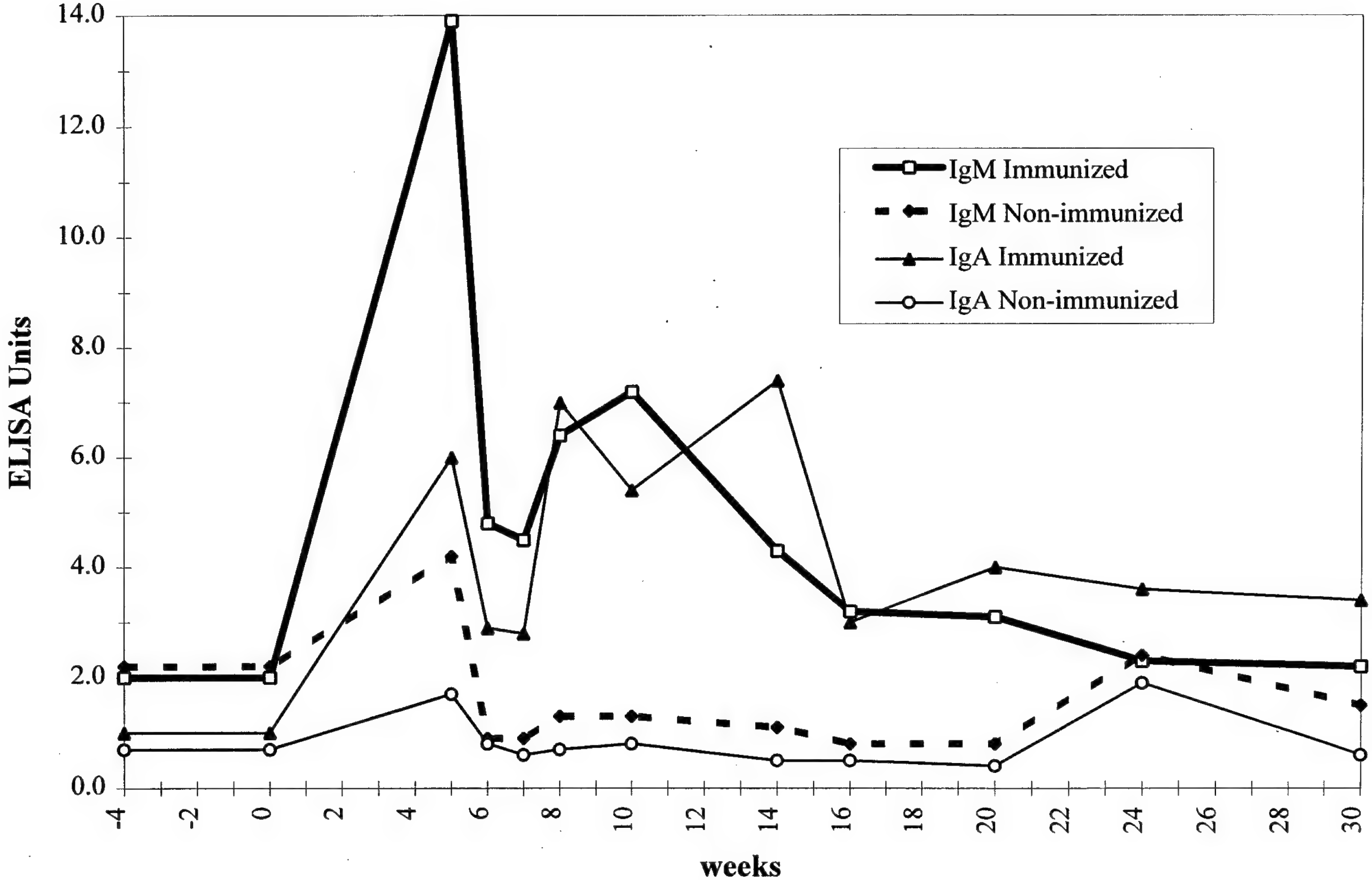
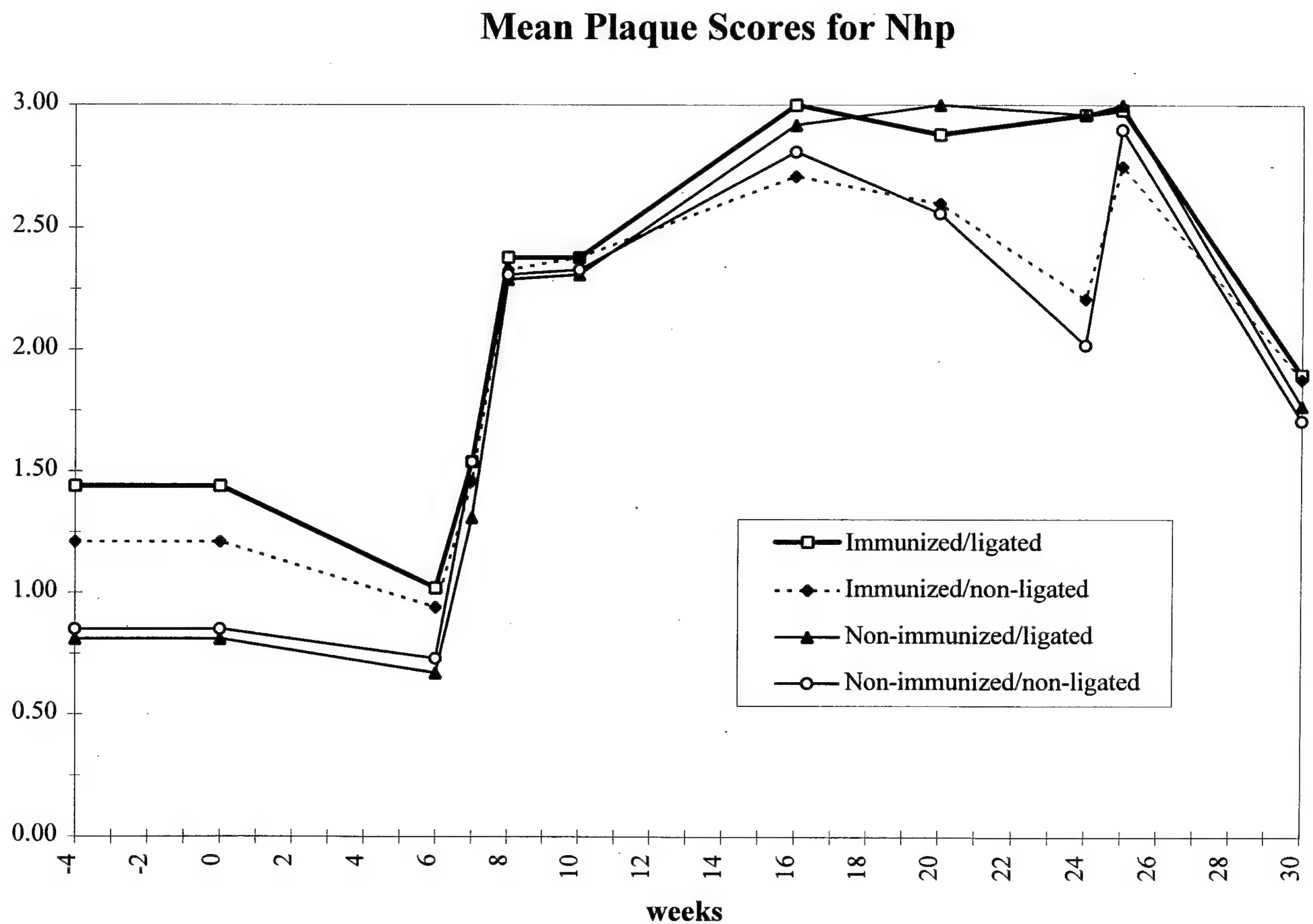


Figure 11: Mean Plaque Scores for Nhp. Depicted are mean group plaque scores for all ligated and non-ligated sites in the Nhp using the plaque index of Löe and Silness. Significant increases in plaque scores were noted with the onset of the gingivitis phase of the study at week 6. Ligatures were placed on sextant 3 experimental teeth at week 12 and were removed at week 25.


Mean Plaque Scores for Nhp
3.00
2.50
2.00
1.50
1.00
0.50
0.00
-4
-2
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
weeks
Immunized/ligated
Immunized/non-ligated
Non-immunized/ligated
Non-immunized/non-ligated

of the immunized ($P < 0.05$) and non-immunized groups ($P = 0.0006$). A similar unexpected increase was noted in the non-ligated sites in the control animals ($P < 0.05$). When comparing ligated to non-ligated sites within each group, the immunized animals showed significantly more plaque in the ligated sites occurring at 24 weeks ($P < 0.003$), while the non-immunized group showed significant differences at 20 ($P < 0.03$) and 24 weeks ($P < 0.006$).

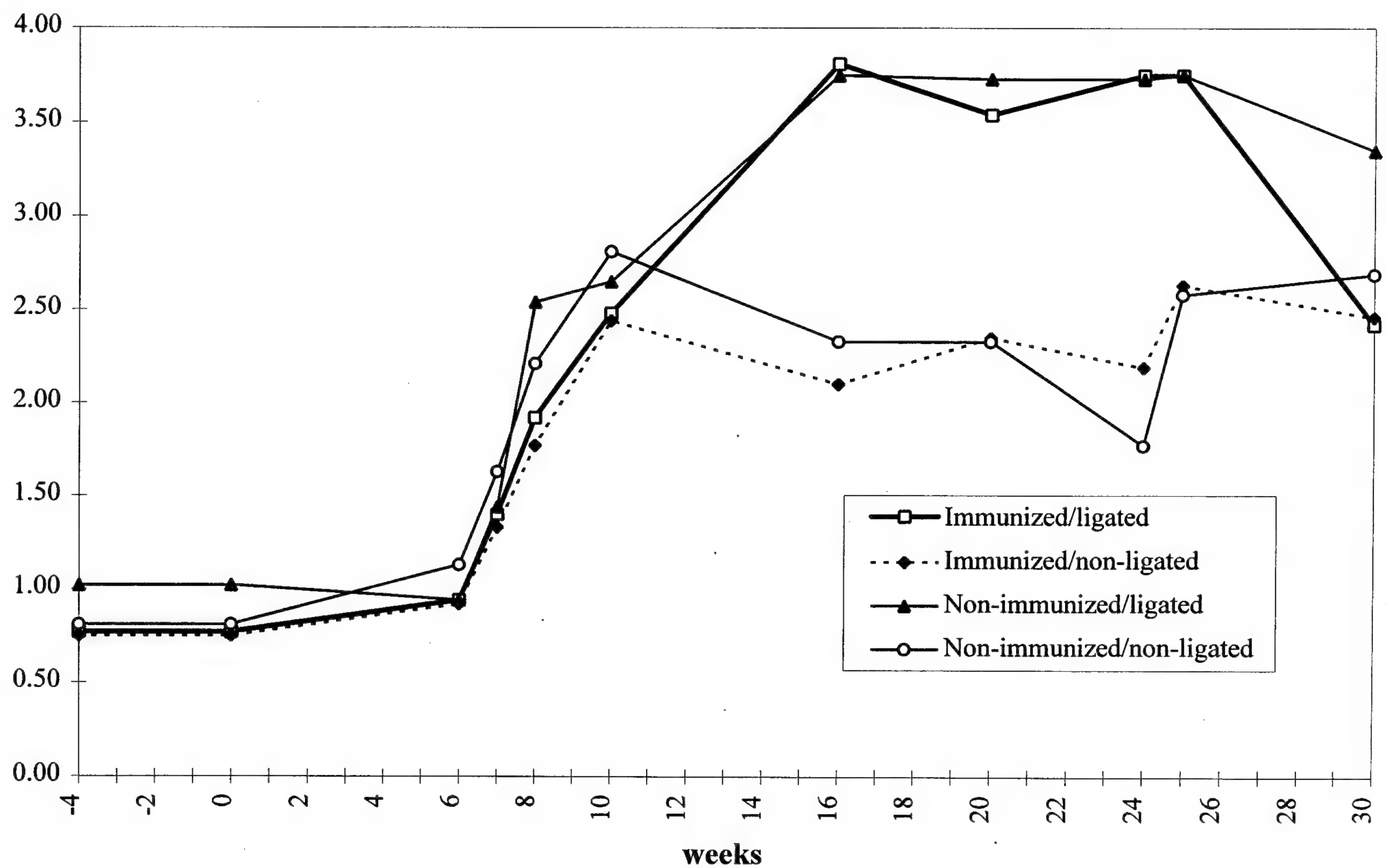
No other significant differences in plaque accumulation were noted between the groups that would attest to an obvious effect of the immunization on this disease parameter.

b. Bleeding Scores

Mean bleeding scores for all sites in both groups are illustrated in Figure 12. Significant increases in bleeding upon probing were seen at all sites with the onset of the gingivitis phase, with all sites demonstrating significant elevations in this parameter from baseline out to 30 weeks. When compared to bleeding scores during the gingivitis phase, ligature placement was accompanied by a significant increase in bleeding in the immunized group at 24 weeks ($P < 0.04$) and in the control group at 20 weeks ($P < 0.002$). Ligature placement was also associated with significantly higher bleeding scores when compared to respective unligated sites within each group.

Figure 12: Mean Bleeding Scores for Nhp. Depicted are mean group bleeding scores for all ligated and non-ligated sites using a modified bleeding index (Appendix A). Significant increases in bleeding scores were noted with the onset of the gingivitis phase of the study at week 6. Ligatures were placed on sextant 3 experimental teeth at week 12 and were removed at week 25.

Mean Bleeding Scores for Nhp



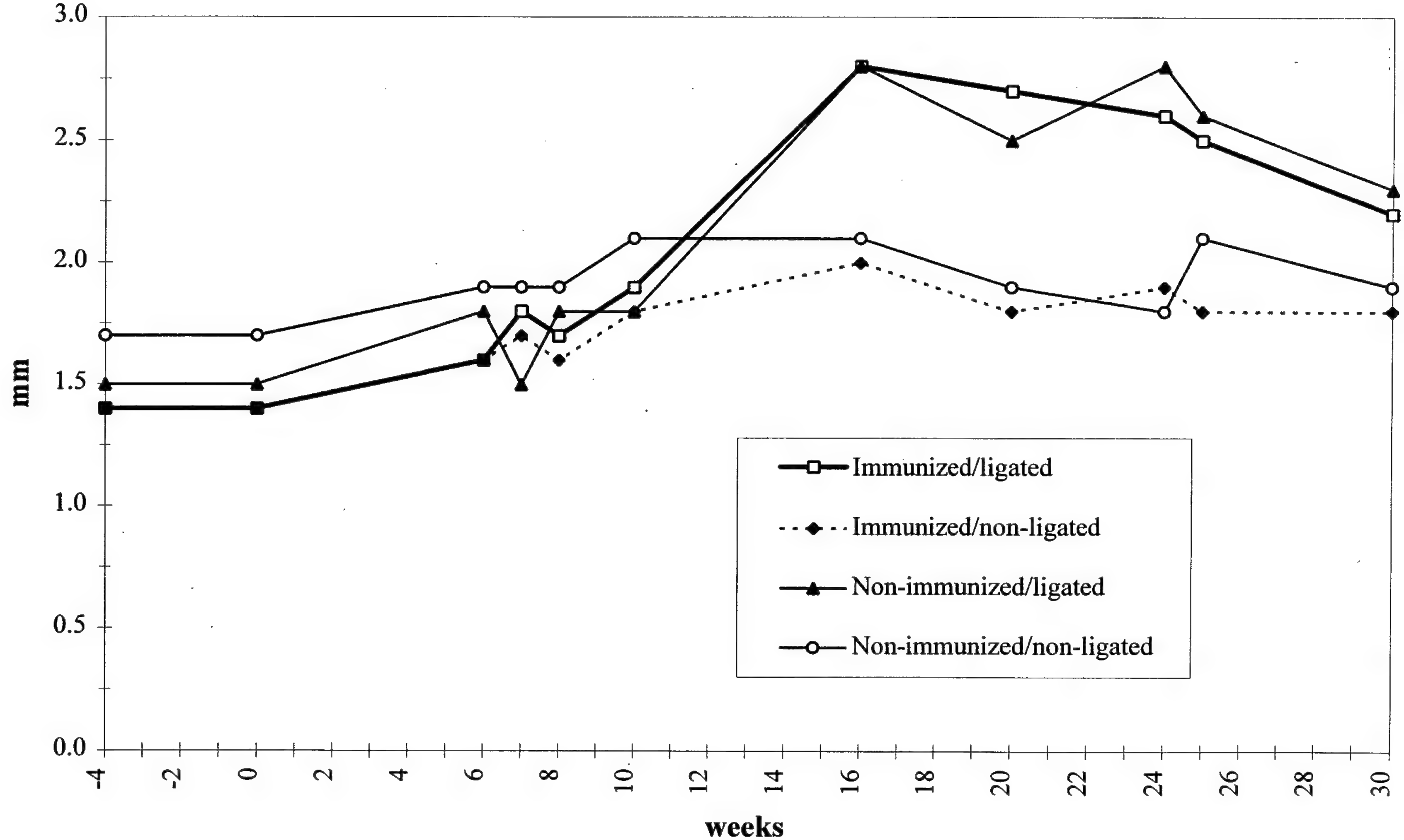
No significant differences were noted in bleeding scores in either ligated or non-ligated sites between the immunized and non-immunized groups to suggest overt association between immunization and this disease parameter.

c. Probing Depths

Mean probing depth measurements for both groups are presented in Figure 13. Ligated sites in both groups showed significant increases in probing depth from baseline values beginning at 10 weeks, coinciding with the end of the gingivitis phase. Ligated sites in the immunized group remained significantly deeper until week 30 (from $P < 0.005$ to $P = 0.0002$) and similarly in the non-immunized group (from $P < 0.007$ to $P < 0.0001$). By week 16, ligature placement in both the immunized ($P = 0.0002$) and control groups ($P = 0.0001$) had caused significant increases in probing depths compared to 10 week measurements. Upon ligature removal, experimental sites in both groups experienced some non-significant probing depth reduction compared to the end of the periodontitis phase, however these sites did continue to exhibit probing depths significantly greater than baseline values out to 30 weeks. As anticipated, non-ligated sites in both groups showed essentially no changes. Within group comparisons of ligated to non-ligated sites revealed ligated sites demonstrating significantly greater probing depths than non-ligated sites in both the immunized (from $P < 0.04$ to $P < 0.002$) and non-immunized groups (from $P = 0.03$ to $P = 0.0001$).

Figure 13: Mean Probing Depths for Nhp. Depicted are mean group probing depth measurements in millimeters (mm) for all ligated and non-ligated sites. Significant increases in probing depths in both Nhp groups were noted in ligated sites beginning at week 10, coinciding with the end of the gingivitis phase. Ligatures were placed on sextant 3 experimental teeth at week 12 and were removed at week 25.

Mean Probing Depths for Nhp



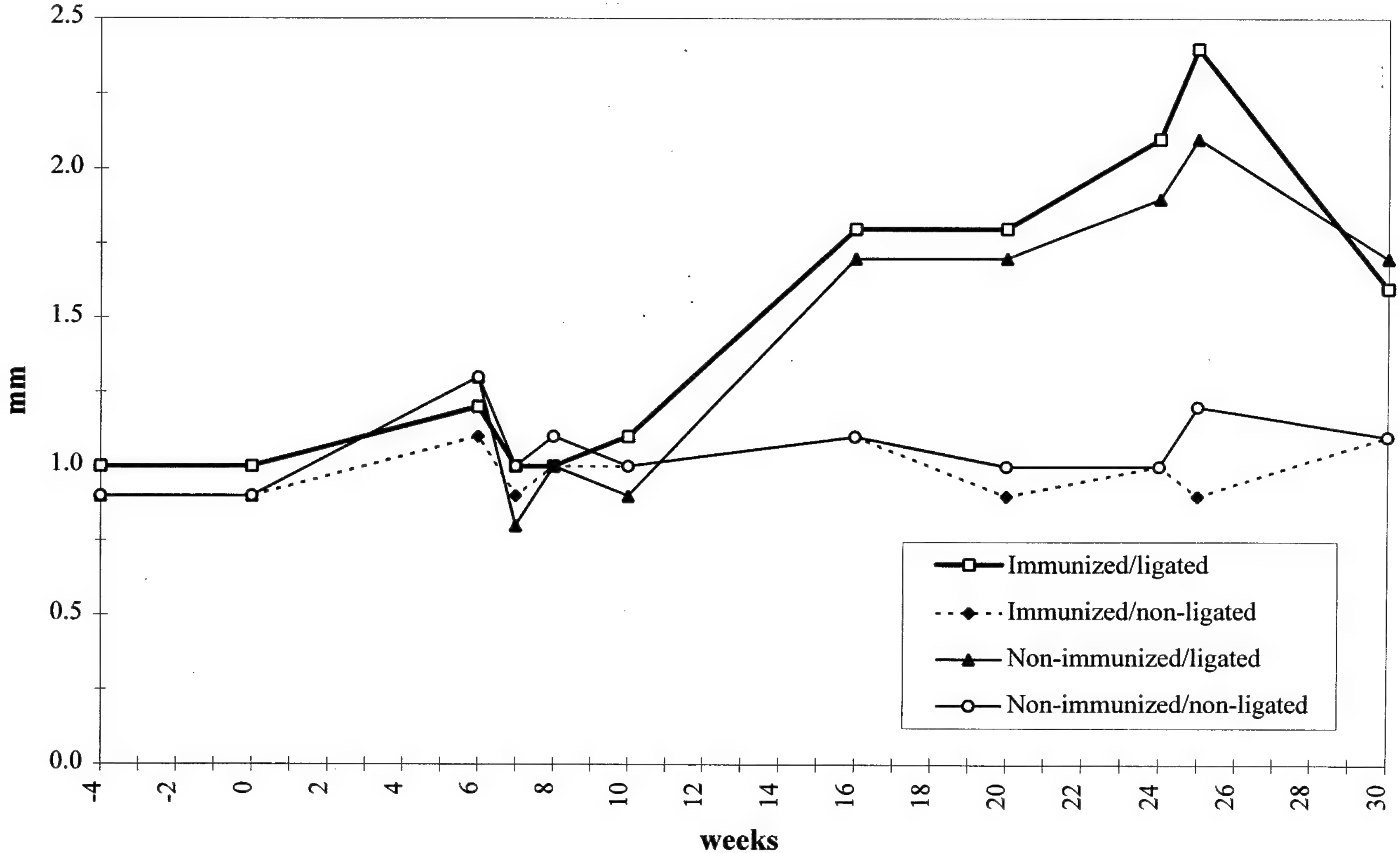
No significant differences in probing depth parameters were found when comparing the ligated and non-ligated sites between groups, again revealing little impact of the immunization on this periodontal disease parameter.

d. Clinical Attachment Levels

Mean attachment level measurements for all sites in both groups are summarized and displayed in Figure 14. Overall, no significant changes in attachment levels were noted in non-ligated sites, as was expected. Ligated sites in both groups demonstrated significant attachment loss from baseline beginning at week 16 with attachment loss remaining significant through week 25 for the immunized group (from $P < 0.05$ to $P < 0.009$), and through week 30 for the non-immunized group (from $P < 0.002$ to $P = 0.001$). Maximal attachment loss for the immunized group was 1.4 mm, for the non-immunized group 1.2 mm. For the immunized and control groups, significant attachment loss occurred from the end of the gingivitis phase to the first post-ligation measurement at week 16 (both $P < 0.001$). With ligature removal in the immunized group, significant gain of attachment level occurred from week 25 to week 30 ($P < 0.03$) with no significant difference in the 30 week value compared to baseline. With ligature removal in the non-immunized group, significant attachment gain also occurred at week 30 ($P = 0.009$), and this value continued to represent significant attachment loss compared to baseline ($P < 0.001$). Ligated sites demonstrated significantly greater attachment

Figure 14: Mean Clinical Attachment Levels for Nhp. Depicted are mean group clinical attachment level measurements in millimeters (mm) for all ligated and non-ligated sites. Significant loss of clinical attachment in both Nhp groups was noted in ligated sites beginning at week 16. Ligatures were placed on sextant 3 experimental teeth at week 12 and were removed at week 25.

Mean Clinical Attachment Levels for Nhp



loss than non-ligated sites from weeks 16 to 30 in the immunized group (from $P < 0.04$ to $P < 0.001$) and from 16-25 weeks in the non-immunized group (from $P < 0.03$ to $P < 0.09$). No significant difference in mean attachment levels between ligated and non-ligated sites remained at week 30 in the non-immunized group.

As noted in the results for the preceding clinical periodontal disease parameters, no significant differences in attachment levels connoting a significant clinical impact of the immunization on this particular measurement were noted.

e. Study of the Effect of the Ligature on Probing Depth and Attachment Level Measurements

An additional assessment was undertaken to determine the possible impact of the 3-0 silk ligatures used in this study on the ability to probe accurately and thus directly influence probing depth as well as attachment level measurements. After all sites were probed and all other clinical data gathered at week 25, ligatures were removed. After 15 minutes, all sites previously anchoring ligatures were reprobed using the same manual periodontal probe by the same examiner. There were no significant differences noted in pre and post-ligature removal probing depths for the immunized group (2.5 ± 0.3 mm pre; 2.6 ± 0.3 mm post) or for the non-immunized group (2.6 ± 0.5 mm pre; 2.6 ± 0.4 mm post) suggesting minimal to no impact of this diameter ligature upon probing depth and subsequent attachment level measurements.

2. CADIA Results

Mean CADIA values for radiographic bone density changes are shown in Figure 15. CADIA revealed no significant interproximal bone density changes in non-ligated sites for any time point analyzed. There were also no significant changes seen during the gingivitis phase for any sites in either group.

Ligature placement produced progressively increasing levels of bone density loss until week 24 at which time maximal bone loss was manifested by both groups. There was a non-signficant greater mean maximal bone loss noted for the non-immunized group at week 24 (-13.07 ± 9.51; CADIA units ± SD) compared to the immunized group (-9.41 ± 6.18; CADIA units ± SD). Point-to-point comparisons as well as grouped comparisons of "total gingivitis values" and "total periodontitis" values of ligated and non-ligated sites were not able to demonstrate any statistically significant differences between the groups by either parametric or non-parametric statistical analyses. At no time point did either group show significantly greater mean bone loss in ligated sites when compared to respective baseline values. After ligature removal at week 25, a non-significant increase in bone density occurred in ligated sites for both groups.

Within each group, using point-to-point comparisons, CADIA demonstrated significantly greater mean bone loss when comparing ligated sites to their respective non-ligated, contralateral control sites. The degree of significance of this mean maximal bone loss difference was much greater in the control group ($P < 0.001$) than in the immunized group ($P = 0.043$). Grouped comparisons of "total periodontitis" values in ligated and their

Figure 15: Mean CADIA Values for Nhp. Shown are mean group radiographic bone density changes in interproximal osseous crestal areas as determined by Computer Assisted Densitometric Image Analysis for all all ligated and non-ligated sites. Values are expressed in CADIA units indicated on the y-axis. Negative values denote loss of radiographic bone density. Ligatures were placed on sextant 3 experimental teeth at week 12 with ligature removal at week 25.

respective non-ligated control sites using the Mann-Whitney U Test showed a 10 fold greater degree of significance for bone density loss in the non-immunized control group ($P = 0.000002$) when compared to the same test for the immunized group ($P = 0.00003$).

Since it was noted that most of the Nhp used in this study exhibited some visual radiographic bone loss at baseline, analysis was done to determine whether sites with lower initial values of crestal alveolar bone density were more prone to further breakdown than those sites that did not manifest similar radiographic evidence of prior destruction. Using the Spearman Rank Order Correlations Test comparing "total periodontitis" CADIA values in ligated sites in both groups to their baseline values, it was found that no relationship of pre-existing bone loss to further bone loss existed in the ligated sites in the immunized group of Nhp. However, a significant relationship was apparent between pre-existing and further bone loss in the same sites of the non-immunized control group ($P < 0.03$).

C. Microbiological Results

1. Effects of Immunization on *P. gingivalis*

The effect of active immunization on the emergence of *P. gingivalis* in the experimental and control sites of the Nhp is characterized in Figures 16 and 17. No significant changes were noted for mean numbers of *P. gingivalis* in ligated or non-ligated sites in either group during the gingivitis phase from weeks 6-10. However, the non-immunized control group showed an increase of approximately 3-fold in *P. gingivalis* levels beginning at week 10, the end of the gingivitis phase.

Figure 16: ***P. gingivalis*** **Emergence in Ligated Sites: Immunized Versus Non-immunized Groups.** The bar graph depicts the mean subgingival levels of *P. gingivalis* reached in ligated sites in both Nhp groups as determined by "Checkerboard" DNA hybridization. All values are expressed times 10^5 as indicated on the y-axis. The asterisks denote significance from baseline values. Ligatures were placed at week 12 and removed at week 25.

P. gingivalis Emergence in Ligated Sites: Immunized Versus Non-immunized Groups

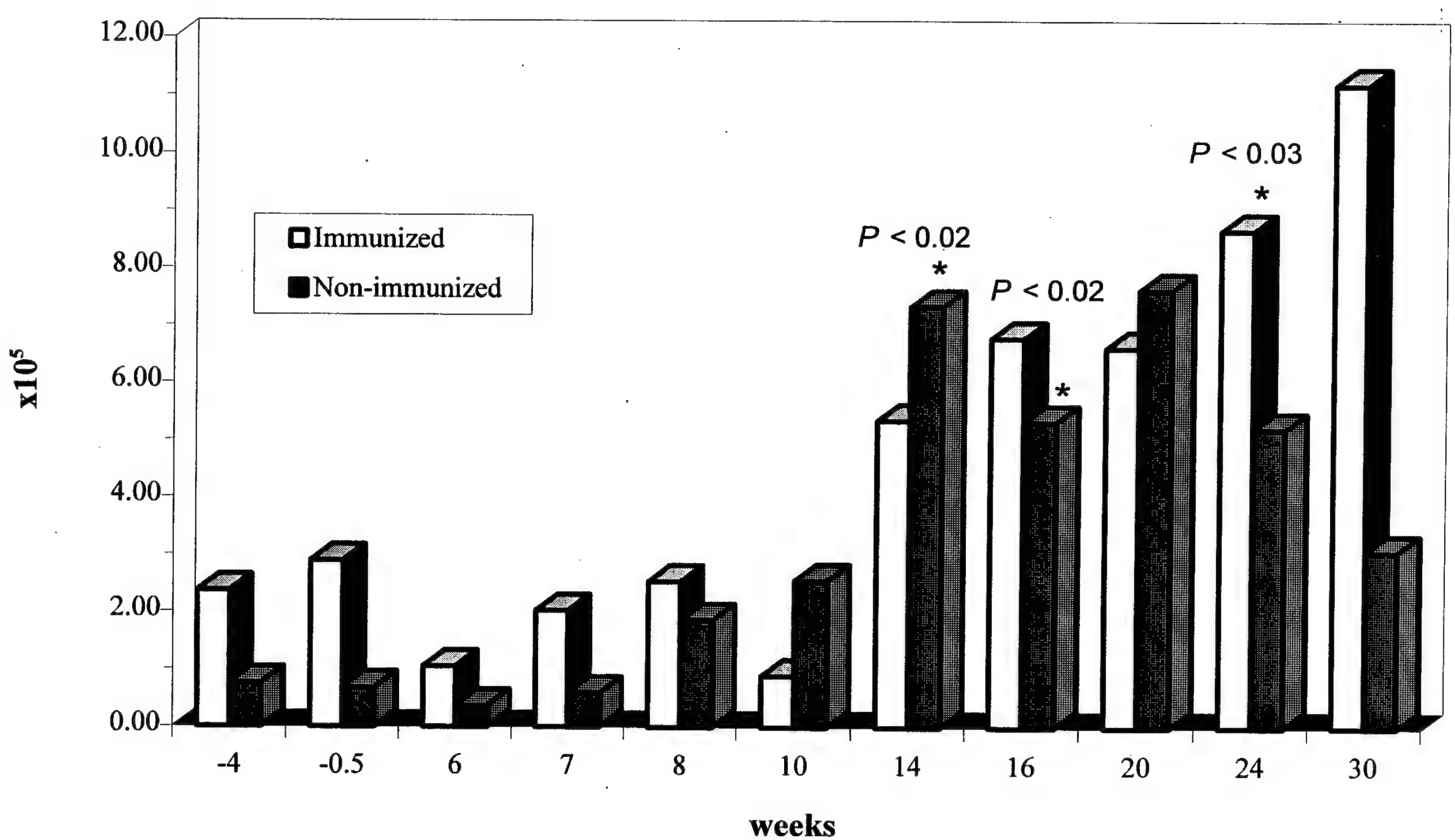
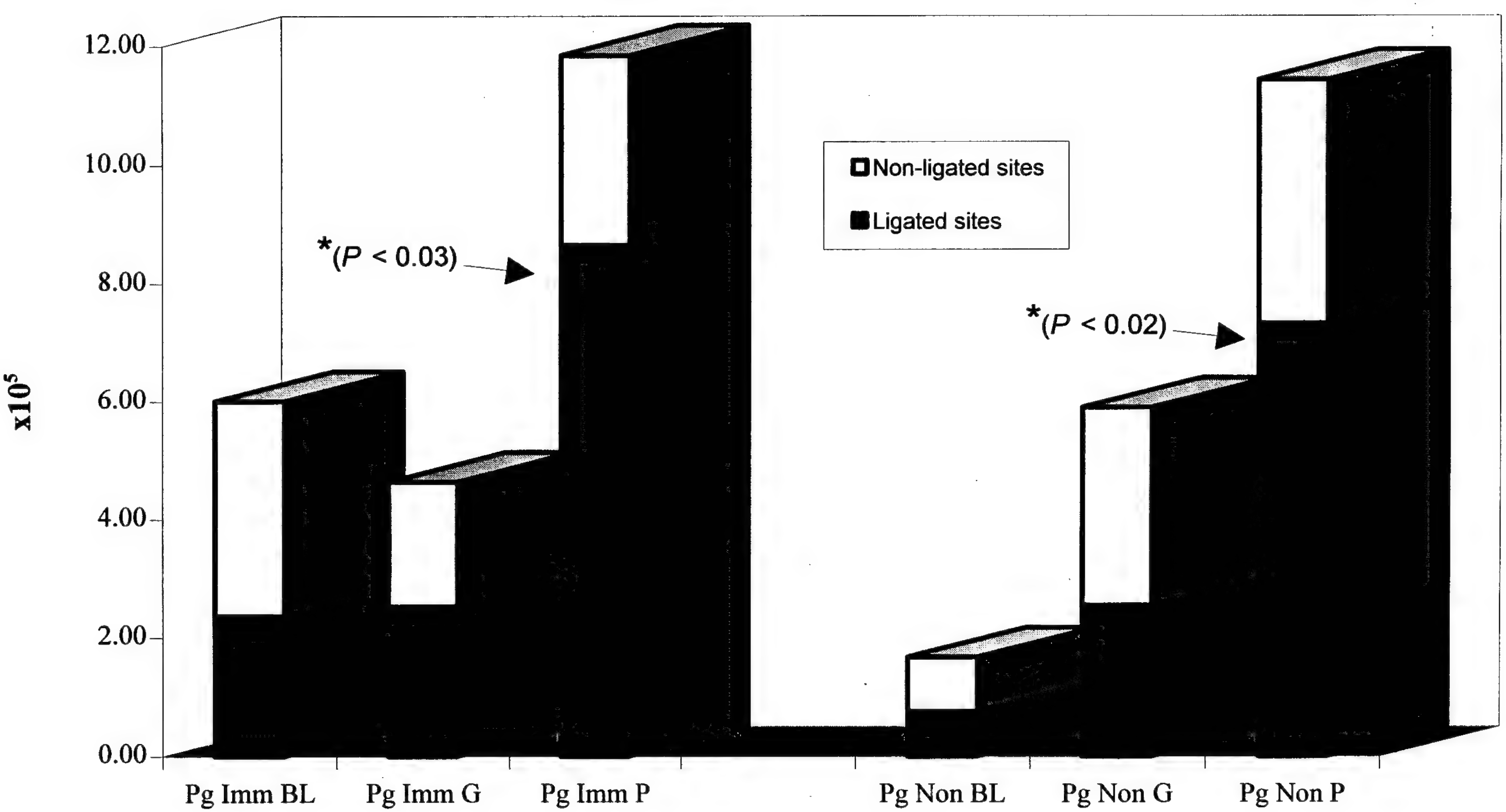


Figure 17: *P. gingivalis* Emergence: Immunized Versus Non-immunized Nhp. The bar graph depicts the maximum mean subgingival levels of *P. gingivalis* reached at baseline (BL), and during the gingivitis (G) and periodontitis (P) phases in ligated and non-ligated sites in both immunized (Imm) and non-immunized (Non) Nhp groups as determined by "Checkerboard" DNA hybridization. All values are expressed times 10^5 as indicated on the y-axis. The asterisks denote significance from baseline values.

P. gingivalis Emergence: Immunized Versus Non-immunized Nhp



Ligature placement was associated with increased mean numbers of detectable *P. gingivalis* from baseline levels which remained elevated beyond ligature removal in both groups. Ligated sites in the immunized group displayed the only significant elevation in *P. gingivalis* at week 24 (12 weeks after ligature placement) ($P < 0.03$). This value was approximately 3-fold greater than baseline levels. There were no significant increases noted between any value during the periodontitis phase compared to final levels attained during the gingivitis phase. *P. gingivalis* levels also reached significance at both 2 weeks ($P < 0.02$) and 4 weeks ($P < 0.02$) after ligation in corresponding sites in the non-immunized group. The maximum response here was approximately 9-fold greater than mean baseline levels. A significant increase in *P. gingivalis* levels occurred from final gingivitis values at week 24 ($P < 0.03$).

Non-ligated sites in neither group showed significant increases in *P. gingivalis* levels during the study. However, a trend toward increasing levels during experimental gingivitis and periodontitis was noted in these sites in the non-immunized group.

Comparing ligated to non-ligated sites within groups, no significant differences were noted at baseline for either group. With disease progression in the immunized group, this comparison yielded no significant differences, although mean total *P. gingivalis* counts were approximately 3 to 8-fold higher after ligature placement in the ligated sites than in the non-ligated sites. This same comparison in the non-immunized Nhp group showed a significantly greater amount of *P. gingivalis* in ligated sites at week 14 only ($P < 0.02$).

Between group comparisons of *P. gingivalis* levels at all ligated and non-ligated sites demonstrated no significant associations or differences.

Additionally, *P. gingivalis* levels as a percentage of the total Gram-negative microbiota examined in this study (12 species) were analyzed for ligated sites. At baseline, *P. gingivalis* constituted 1.9% of the total Gram-negative microbiota in the immunized group, and 0.9% in the non-immunized group. With maximal emergence of *P. gingivalis* after ligature placement, this microorganism represented 5.9% of the total analyzed Gram-negative microbiota in immunized, ligated sites, and 7.1% in the same sites of the non-immunized Nhp.

2. Effects of Immunization on Emergence of Subgingival Microbiota

Results demonstrating the effect of active immunization on the emergence of 15 additional Gram-positive and Gram-negative bacterial species analyzed in this study are summarized in Figures 18-22. Immunization as well as the onset of experimental periodontal disease was accompanied by various diverse patterns of emergence in the microorganisms evaluated. Significant mean maximal changes from baseline values were noted for various bacteria in ligated and non-ligated sites during the gingivitis and periodontitis phases.

When emergence patterns of the 12 different Gram-negative anaerobic species were examined in ligated sites, it was determined that 1 out of 12 species became significantly elevated above baseline during the gingivitis phase in the immunized group, whereas 5 out of 12 species became significantly elevated in the non-immunized group.

Figure 18: Emergence of Microorganisms in Immunized Nhp. The bar graph depicts the maximum mean subgingival levels of test microorganisms present at baseline (BL), and attained during the gingivitis (G) and periodontitis (P) phases in both ligated and non-ligated sites in the immunized Nhp group as determined by "Checkerboard" DNA hybridization. All values are expressed times 10^5 as indicated on the y-axis. The asterisks denote significance from baseline values. Test microorganisms include *P. gingivalis* (Pg), *A. actinomycetemcomitans* (Aa), *F. Nucleatum* (Fn), *C. rectus* (Cr), *B. forsythus* (Bf), *S. sanguis* (Ss), and *S. intermedius* (Si).

Emergence of Microorganisms in Immunized Nhp

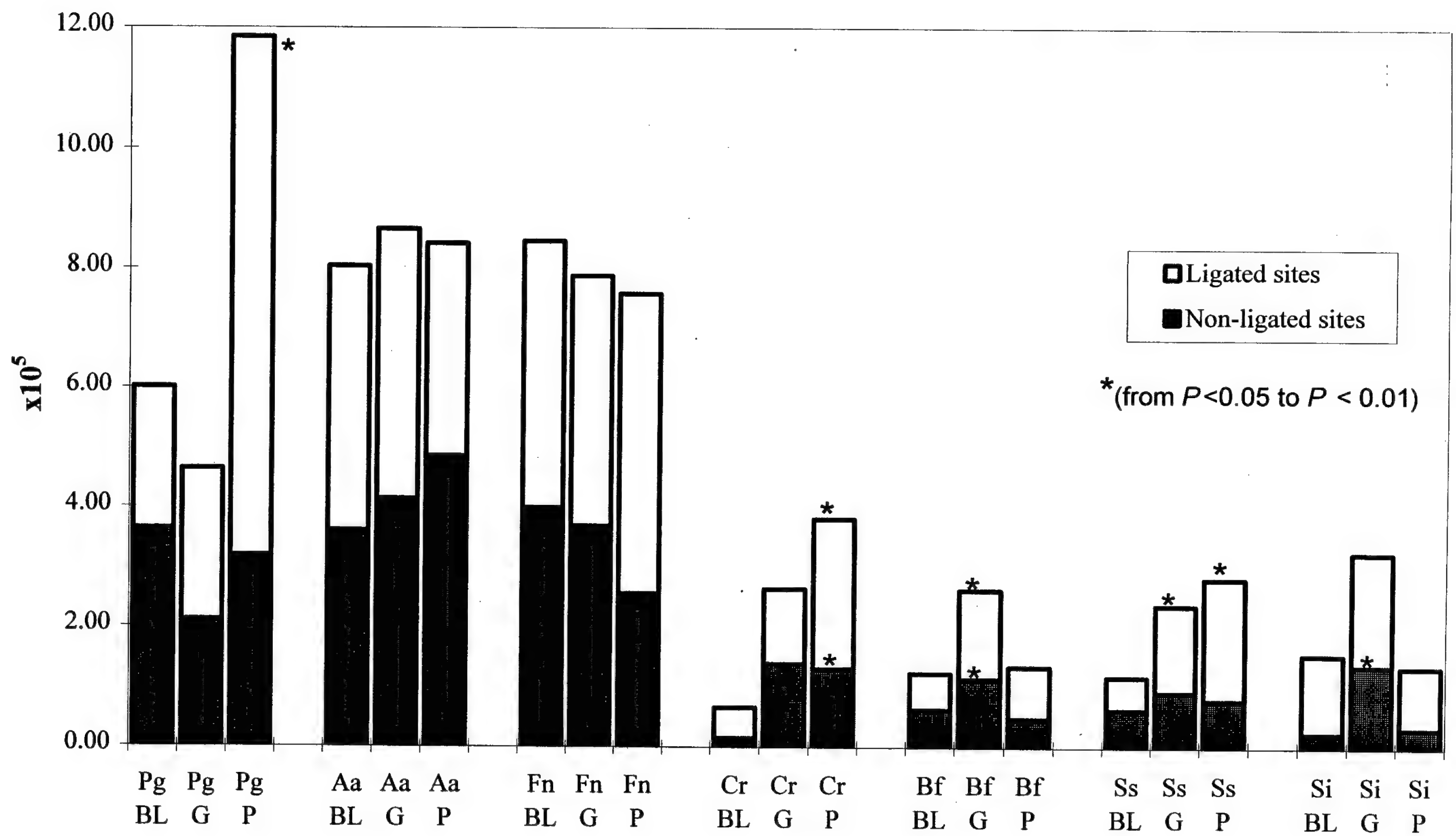


Figure 19: Emergence of Microorganisms in Immunized Nhp. The bar graph depicts the maximum mean subgingival levels of test microorganisms present at baseline (BL), and attained during the gingivitis (G) and periodontitis (P) phases in both ligated and non-ligated sites in the immunized Nhp group as determined by "Checkerboard" DNA hybridization. All values are expressed times 10^5 as indicated on the y-axis. The asterisks denote significance from baseline values. Test microorganisms include *T. denticola* (Td), *P. nigrescens* (Pn), *C. showae* (Cs), *S. gordonii* (Sg), *E. corrodens* (Ec), *C. gracilis* (Cg), and *P. intermedia* (Pi).

Emergence of Microorganisms in Immunized Nhp

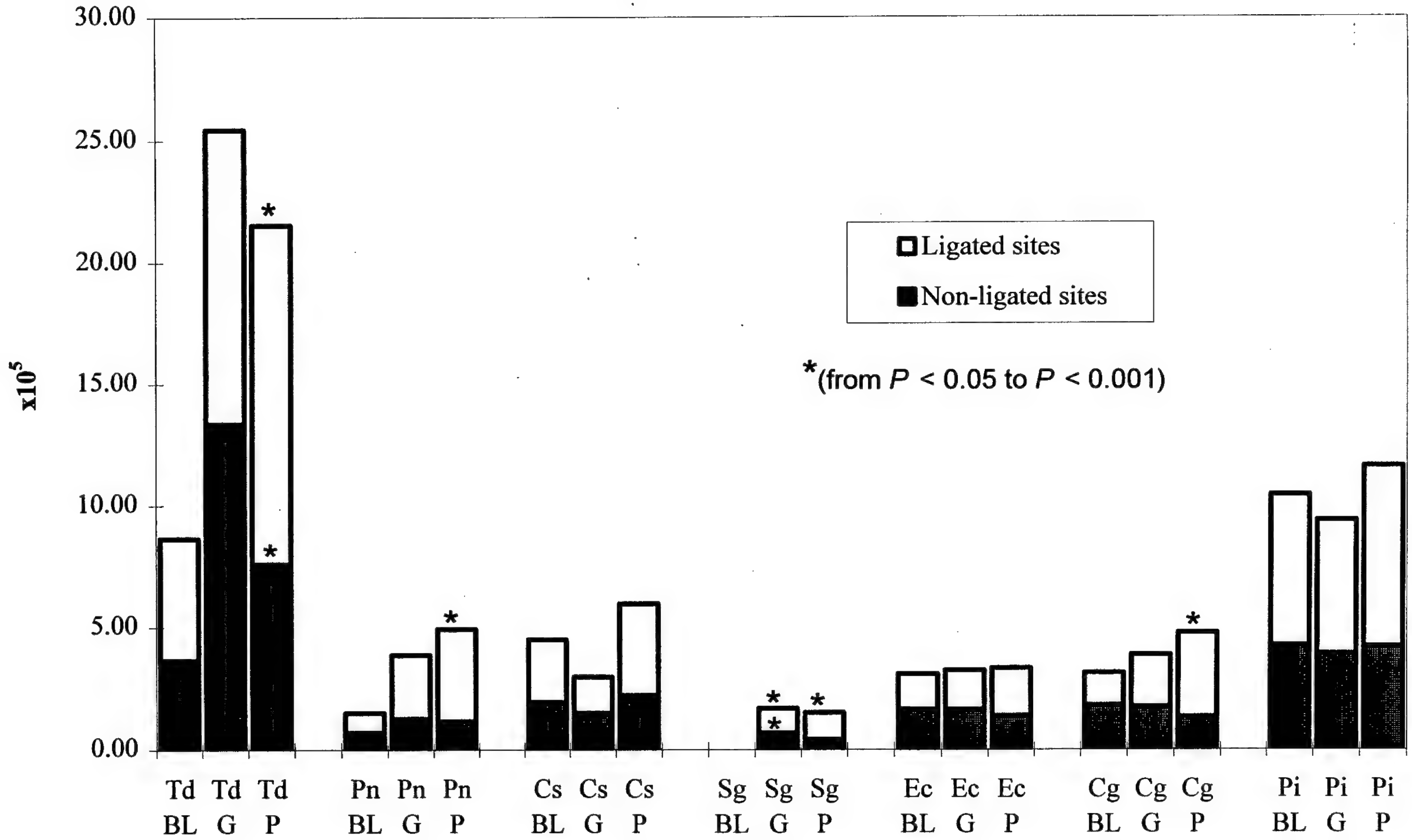


Figure 20: Emergence of Microorganisms in Non-immunized Nhp. The bar graph depicts the maximum mean subgingival levels of test microorganisms present at baseline (BL), and attained during the gingivitis (G) and periodontitis (P) phases in both ligated and non-ligated sites in the non-immunized Nhp group as determined by "Checkerboard" DNA hybridization. All values are expressed times 10^5 as indicated on the y-axis. The asterisks denote significance from baseline values. Test microorganisms include *P. gingivalis* (Pg), *A. actinomycetemcomitans* (Aa), *F. Nucleatum* (Fn), *C. rectus* (Cr), *B. forsythus* (Bf), *S. sanguis* (Ss), and *S. intermedius* (Si).

Emergence of Microorganisms in Non-immunized Nhp

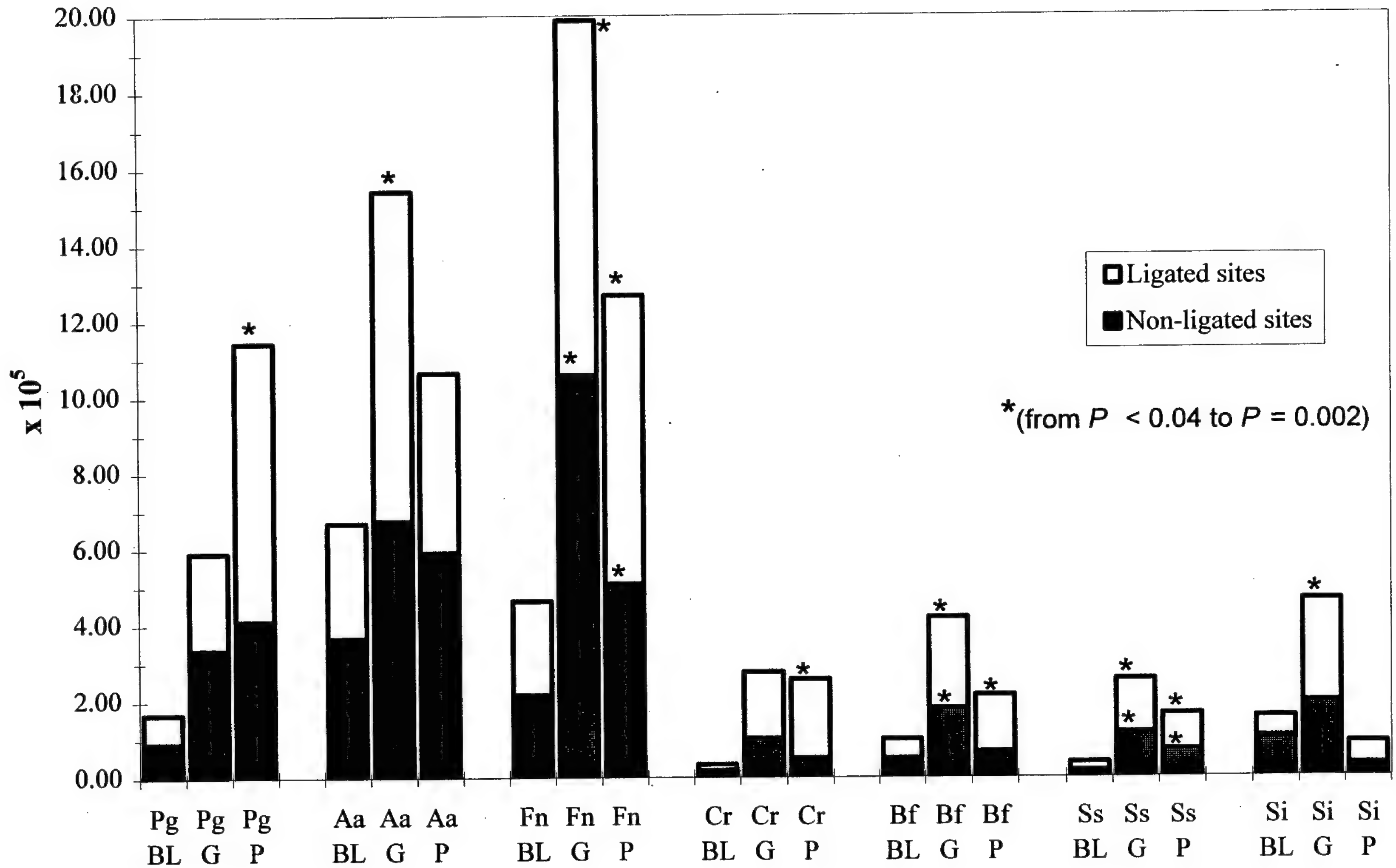


Figure 21: Emergence of Microorganisms in Non-immunized Nhp. The bar graph depicts the maximum mean subgingival levels of test microorganisms present at baseline (BL), and attained during the gingivitis (G) and periodontitis (P) phases in both ligated and non-ligated sites in the non-immunized Nhp group as determined by "Checkerboard" DNA hybridization. All values are expressed times 10^5 as indicated on the y-axis. The asterisks denote significance from baseline values. Test microorganisms include *T. denticola* (Td), *P. nigrescens* (Pn), *C. showae* (Cs), *S. gordonii* (Sg), *E. corrodens* (Ec), *C. gracilis* (Cg), and *P. intermedia* (Pi).

Emergence of Microorganisms in Non-immunized Nhp

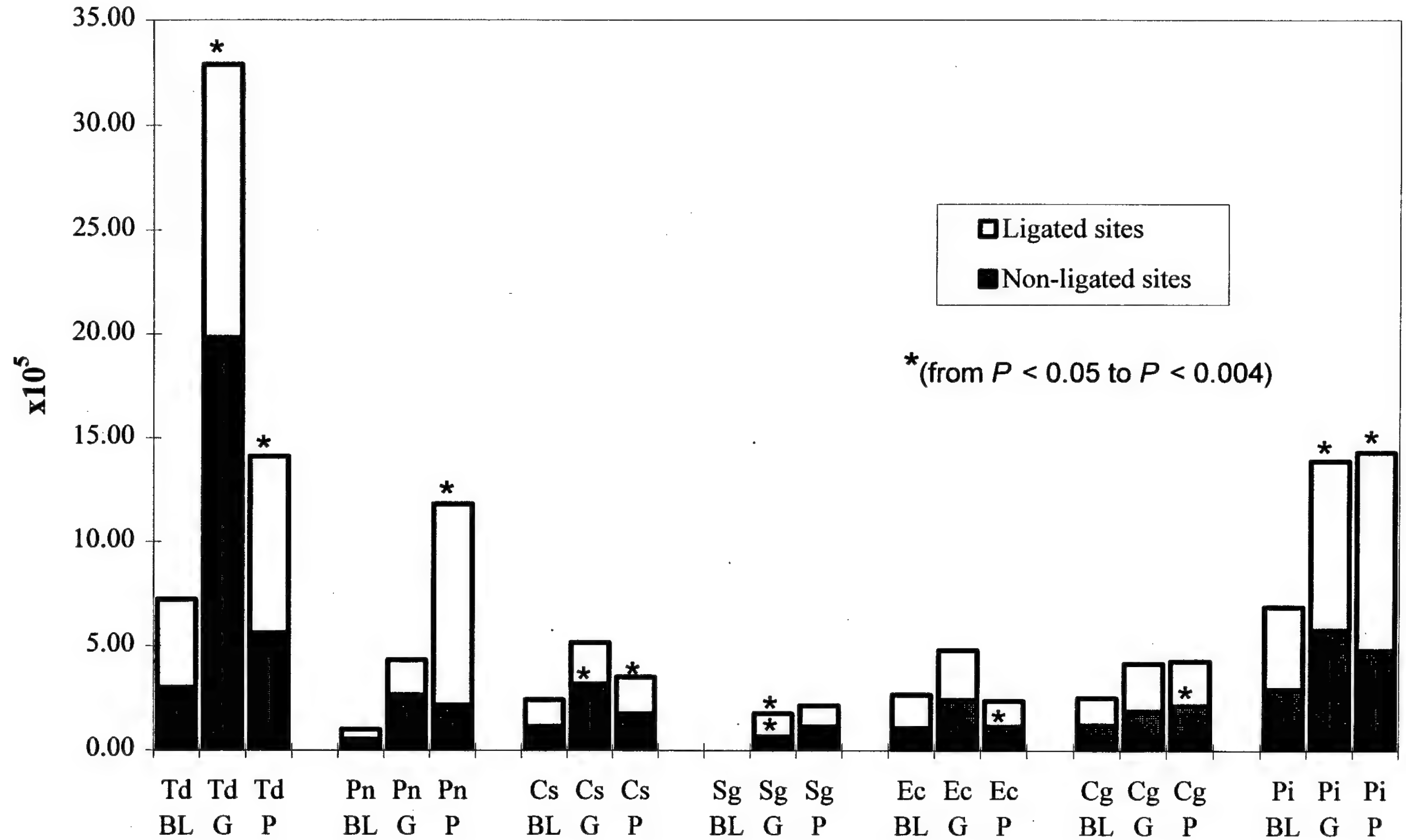
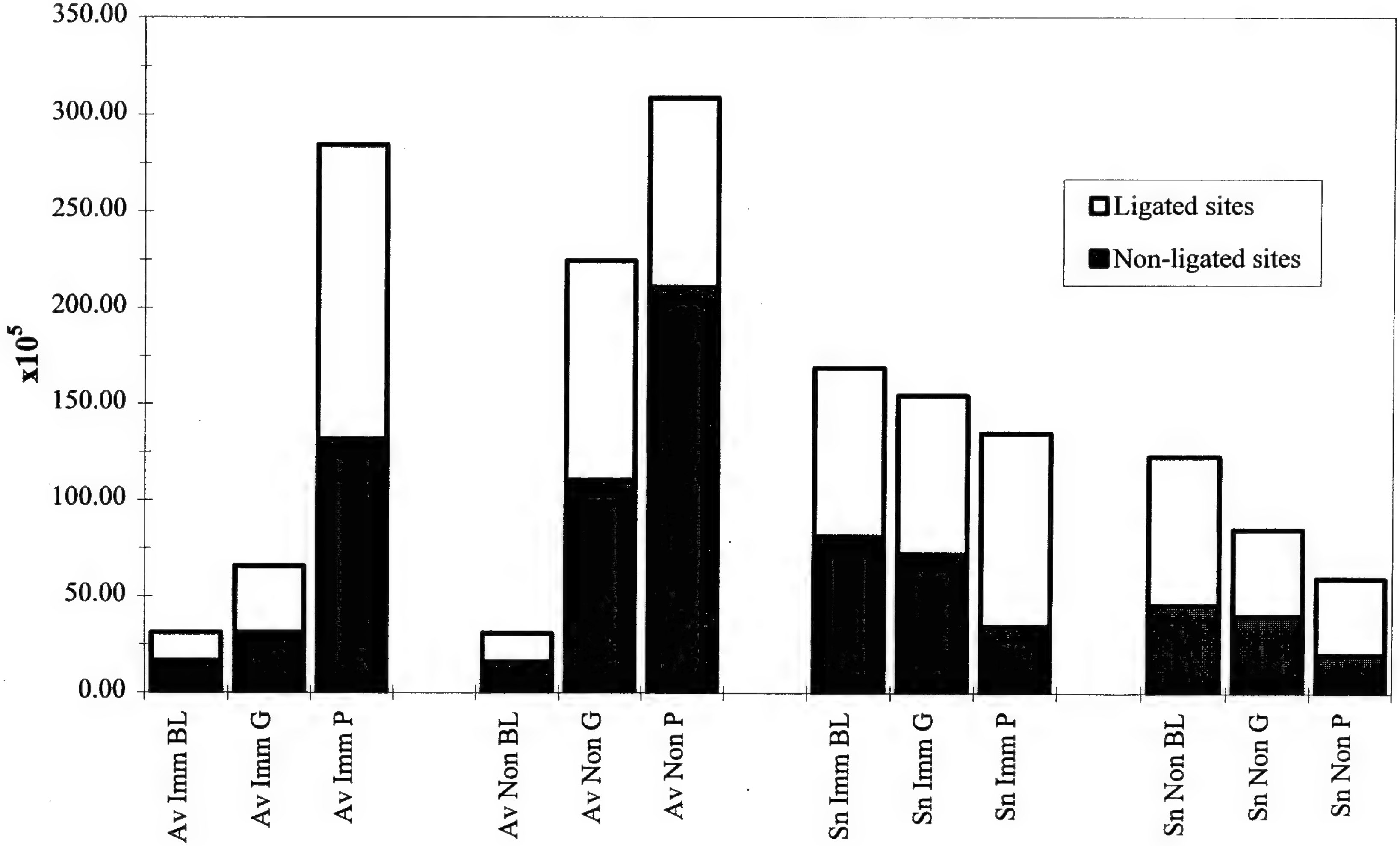


Figure 22: Emergence of Microorganisms in Immunized and Non-immunized Nhp. The bar graph depicts the maximum mean subgingival levels of *A. viscosus* (Av) and *S. noxia* (Sn) present at baseline (BL), and attained during the gingivitis (G) and periodontitis (P) phases in both ligated and non-ligated sites in the immunized (Imm) and non-immunized (Non) Nhp groups as determined by "Checkerboard" DNA hybridization. These microorganisms are included in a separate chart because they reached higher values than other test bacteria, nevertheless were not significantly elevated above baseline values. All values are expressed times 10^5 as indicated on the y-axis.

Emergence of Microorganisms in Immunized and Non-Immunized Nhp



The same comparison for the periodontitis phase yielded 5 out of 12 Gram-negative species becoming significantly elevated above their baseline values for the immunized group, and 8 out of 12 reaching significant levels in the non-immunized group. Thus, 25% more Gram-negative anaerobic species became significantly elevated in the non-immunized group.

Between group comparisons of mean maximal and total mean maximal emerging microorganism counts in ligated and non-ligated sites, however, showed only minor variations, with no significant differences or trends noted. Table 6 shows the ratios of peak levels of microorganisms achieved after ligature placement to their respective baseline levels. These ratios tended to be higher for the non-immunized Nhp group, however, there were no significant differences between the groups.

Table 6

RATIO OF PEAK LEVELS OF MICROORGANISMS ACHIEVED AFTER LIGATURE PLACEMENT TO BASELINE LEVELS

Group	Gram-negative ratio (Mean ratio ± SD)	Gram-positive ratio (Mean ratio ± SD)
Immunized	2.3 ± 1.5*	8.7 ± 6.5@
Non-immunized	5.1 ± 6.2*	13.9 ± 12.9@

*N.S. difference between groups, $P < 0.16$
@N.S. difference between groups, $P < 0.24$

IV. DISCUSSION

Recent studies by Ebersole *et al.*, (1991) and Persson *et al.*, (1994) examining the effect of active immunization on experimentally-induced periodontitis in *M. fascicularis* using inactivated whole-cell *P. gingivalis* as an antigen have been performed, albeit with conflicting results. These studies as well as those previously reviewed delineating the clinical, histological, microbiological, and immunological responses of the cynomolgus monkey to experimental periodontal disease have helped to establish this species as a good model for the study of potential immunological modulation of experimental periodontitis. This modulation could take the form of active immunization which describes the process of stimulating an intact immune system into producing a protective response. Using an appropriate model, the study presented here describes for the first time an attempt to alter the progression of experimentally-induced gingivitis and periodontitis by active immunization with an isolated and purified bacterial virulence factor from a highly implicated periodontal pathogenic bacterial species.

A precedent for the concept of targeting a specific bacterial virulence factor in an effort to prevent disease exists in the administration of the DPT vaccine in humans. The Diphtheria and tetanus bacilli, in this example, possess potent toxins which are neutralized by antibodies produced by the administration of the toxoid components in the DPT vaccine.

The cysteine protease used as the immunogen in this study, porphypain-2, was selected for several reasons. First, it is associated with *P. gingivalis*, a highly pathogenic microorganism that is continually and ubiquitously implicated in advanced destructive forms of periodontitis in humans. Porphypain-2 is a virulence factor which could help explain the grossly destructive

tendencies of this bacterium. Secondly, the results of Persson *et al.*, (1994) demonstrated that although a reduction of *P. gingivalis* through immunization using whole cells of *P. gingivalis* was attained (non-significant) and alveolar bone loss was significantly reduced, nevertheless the clinical progession of ligature-induced disease was not completely inhibited. Thus, one could hypothesize that factors other than the mere reduction in numbers of a pathogenic bacterial species may be necessary to eliminate destruction in the subgingival microenvironment, such as preventing the emergence of other pathogenic microbial species or inhibiting the activity of bacterial virulence factors that may be inducing or interfering with host immune responses. Additionally, porphypain-2 has been purified and isolated, by Lantz *et al.*, (1993), and made readily available in a form appropriate for use as an immunogen. This same group has characterized this protease to the degree that its plasmin-like, fibrinogenolytic activity could help explain a major destructive weapon available to *P. gingivalis*.

The nonhuman primates used in this study demonstrated serum IgG, IgM, and IgA antibodies to both the whole cells of *P. gingivalis* and to the protease porphypain-2 prior to active immunization. The baseline levels of antibodies determined by ELISA showed a broad range of values, indicating not only past exposure to the antigens, but also demonstrating what may be an overriding consideration in any studies in which attempts are made to manipulate the immune system, namely that some animals are innately better immunologic responders than others. This ability to respond to a particular antigen is determined genetically within each species, and may be inherited as an autosomal dominant trait (Goodman, 1987). This variability in immune response was overtly manifested in this study by individual Nhp such as #N11 and

#R01 which demonstrated consistently higher antibody levels of each isotype after immunization than other members of that particular group.

In this study, Nhp in the experimental group were immunized with a series of three subcutaneous injections two weeks apart. The results of active immunization showed that a dramatic serum antibody response occurred, predominantly of the IgG isotype. This IgG response was detected by ELISA against the whole cells of *P. gingivalis* as well as to porphypain-2, and remained significantly elevated compared to baseline levels and to the non-immunized control group throughout the study. The IgG response directed at the protease antigen was much more specific, elevated, and sustained compared to the anti-*P. gingivalis* IgG response. The lesser degree of IgG response to the whole cells of *P. gingivalis* may be explained by the myriad number of other antigens that are also present on or near the cell surface of *P. gingivalis*. Specificity testing performed using *A. actinomycetemcomitans* and *P. intermedia* were also used to confirm the specificity of the currently used ELISA test. Evaluation of the kinetics of the IgG responses determined that the highest antibody levels to the whole cells of *P. gingivalis* and to the protease antigen were attained immediately after immunization, remaining significantly elevated, with gradual reductions until study end at 30 weeks.

The initiation of experimental gingivitis and periodontitis with the anticipated emergence of subgingival microorganisms like *P. gingivalis* and the subsequent presence of greater numbers of antigens saw no additional signficant changes in serum antibody levels. The fact that IgG levels produced by the immunization were already very high may have obscured any additional alterations in antibody levels produced by the presence of greater numbers of antigens in the relatively few experimental sites in the Nhp.

Although active immunization caused increases in IgM and IgA levels in the Nhp, these levels soon returned to baseline and at no time reached significance. These results differ from other studies (Ebersole *et al.*, 1991; Giardino, 1991) in which whole-cell *P. gingivalis* immunization produced significantly greater and sustained responses in these two antibody isotypes. Since the adjuvant used in the Ebersole *et al.*, (1991) study was similar to the one used in this study, comparison may be possible and one may speculate that the presence of multiple dominant antigens on the cell surface of *P. gingivalis* made it more likely for a pronounced primary immune response represented by IgM to occur than the one produced in this study by a single protein antigen. The disparity in IgA responses are difficult to speculate on since the function of this antibody in Nhp has yet to be fully determined (Mestecky and McGhee, 1987).

In this study, active immunization with porphypain-2 did not produce a drastic reduction in clinical periodontal disease parameters. In general, clinical parameters of plaque, bleeding on probing, probing depths and clinical attachment levels showed minimal differences that could be attributed to the effect of immunization. The inherent problems associated with probing depth and attachment level measurements aside, it remains to be elucidated what the actual significance of a given amount of attachment loss in this Nhp model represents compared to a similar given amount in the human. It is uncertain what particular threshold level of attachment loss, for instance, needs to be exceeded in order to declare a given amount clinically significant. The Nhp in this study had mean baseline probing depths of approximately 1.5 mm. Maximal probing depths recorded even after 12 weeks of ligation averaged only 2.8 mm. Maximal clinical attachment loss ranged from 1.2-1.4 mm. On such a small scale, using even the most senstitive

periodontal probes presently available, it may difficult to show significant differences between groups using these parameters of periodontal disease.

An interesting additional finding in this study was the lack of influence that the presence of the silk ligatures appeared to have on probing depth measurements and attachment level recordings. It has been suggested by other clinical investigators including Schou *et al.*, (1993) and Persson *et al.*, (1994), that inflammation and ligatures can induce significant errors in probing depth and attachment level assessments. It is clear from our findings that the influence of the ligature as determined from pre- and post-ligature removal probing depth measurements 15 minutes apart appears to be minimal.

Rather than relying upon clinical indices or attachment level measurements to define a gold standard for disease activity, this study sought to use radiographic bone density changes determined by CADIA analysis of standardized longitudinal radiographs to be the true measure of disease progression. The results showed that significant interproximal bone density loss occurred in ligated sites in both study groups when compared to their respective unligated contralateral control sites. Had the immunization worked perfectly, then no difference would have existed in this comparison between the ligated and unligated sites, and a P value greater than 0.05 would have indicated this. The degree of significance of this difference in the immunized group ($P = 0.043$) indicates that the ligated sites in the immunized group were more similar in bone density loss to their unligated control sites than the same comparison between ligated and unligated sites in the non-immunized group ($P < 0.001$).

Nevertheless, when bone density loss in ligated sites was compared betwen groups, there were no statistically significant differences noted. These results may be explained by high

standard deviations accompanying the meaned CADIA measurements, a function of the small sample size of the test groups as well as individual variation in susceptibility to periodontal breakdown.

It was also noted that ligated sites in both groups had still been losing bone at a relatively consistent rate until ligature removal. The slopes of the bone loss curves for each group also differed, with a steeper curve presenting for the non-immunized group. According to the experimental disease model depicted by Kornman *et al.*, (1981), our study was within the window for having achieved maximal bone loss, 12 weeks having elapsed after ligation. Nevertheless, it appears that ongoing bone loss was occurring at the time of ligature removal and it is possible that a significant difference in bone loss between groups may have occurred were the ligation phase to have been continued beyond that point.

Of additional interest is the gain in bone density that was seen 5 weeks after ligature removal. No mechanical oral hygiene was performed, but the Nhp were converted back to a normal hard diet. Our results indicate that this animal model demonstrates an ability to undergo a spontaneous regeneration of interproximal crestal bone density upon ligature removal. A similar gain in clinical attachment level after ligature removal was also noted in this study. If periodontitis implies an irreversible loss of soft and hard tissue attachment to the tooth, then proper comparison between groups might entail analysis of that periodontal destruction that is truly permanent. In the case of the cynomolgus monkey, it is suggested to compare these results after ligature removal followed by an adequate rebound period, which remains as yet undetermined.

Perhaps the most significant findings associated with active immunization in this study are suggested by the microbiological results based on state of the art DNA hybridization technology and computerized densitometric analysis of the resultant blots. Returning to the example of the DPT vaccine, the toxoid in the vaccine may neutralize the diptheria and tetanus toxins and yet not necessarily prevent colonization by these microorganisms. Similarly, it was noted in this study that the emergence of *P. gingivalis* was not prevented or suppressed by immunization. This may have been due to the production of antibodies of lesser avidity or poor opsonic properties, or the possibility that porphypain-2 or other proteases were able to neutralize the antibodies. At no point was a significant inverse correlation of antibody levels with levels of *P. gingivalis* or any of the other microorganisms evident. It was hypothesized that *P. gingivalis* would be inhibited or eliminated because its nutrient requirements as an asaccharolytic organism may have been highly dependent upon the activities of porphypain-2. It was also considered that since the protease is intimately associated with the *P. gingivalis* cell surface, antibody activities directed toward the protease may increase opsonization and lead to increased elimination of *P. gingivalis* by phagocytic immune cells. This study did not determine the specific IgG subclasses produced by immunization, although it has been shown that IgG1 is the prodominant subclass produced in response to protein antigens, with lesser increases in IgG3 and IgG4 levels (Hammarstrom and Smith, 1986). Ishizaka *et al.*, (1967) showed that IgG1 and IgG3 avidly fix complement. It is possible to consider that assuming adequately functioning antibodies to porphypain-2 were produced by immunization and were able to limit or inhibit protease activity, that the lack of overt reduction in clinical inflammation and tissue destruction in the immunized group may have been produced by an offsetting increase in inflammation due to complement

activation. Also, it must be considered that only serum antibodies were analyzed in this study. The effect of parenteral immunization on local antibody production, activity, and interactions in the periodontal pocket where the systemic, local and secretory immune systems merge can only be speculated upon.

Yet, immunization was associated with fewer species of Gram-negative anaerobic microorganisms becoming emergent during both the gingivitis and periodontitis phases of the study. Although the *in-vivo* mechanisms by which the antibody response to porphypain-2 may have exerted an effect on the emergence of other Gram-negative bacteria in the subgingival microecology are unknown, it can be speculated that suppression of protease activities within the periodontal pocket produced by the immunization may have made nutrient factors used for growth by various Gram-negative pathogenic bacteria unavailable and thus inhibited their emergence in the developing destructive lesion of periodontitis.

Since *P. gingivalis* became significantly elevated above baseline values after ligature placement in the immunized animals and clinical parameters of periodontal disease were not significantly reduced in this study, it is possible that other virulence factors associated with *P. gingivalis* or other periodontopathogenic bacteria may be important in contributing to destruction directly, or indirectly by inciting exuberant host immune responses, or by inhibiting host immune responses. It is unlikely that a single bacterial virulence factor is responsible for causing tissue damage. Although that factor may be necessary for tissue destruction to occurr, it is unlikely to be sufficient for disease pathogenesis to result. Nevertheless, an important finding of this study examining the effect of immunization on just one of a potential countless number of virulence factors present at any one moment in the complex subgingival milieu is that immunization

against this particular bacterial protease was associated with a demonstrated alteration in the microbial ecology. This alteration represented a tendency for fewer microorganisms implicated with periodontal tissue destruction to emerge. Preventing or limiting the conversion of the periodontal microbiota to one dominated by Gram-negative anaerobic, pathogenic bacteria may hold the key to limiting periodontal destruction in the future.

It is interesting to contemplate the effects of potentially targeting the most prominent virulence factors from the most commonly implicated periodontopathogenic bacteria. Thus it may ultimately be possible for multi-component vaccines to be developed which incorporate a number of these purified virulence factors. Determination of these virulence factors and further animal testing using greater sample sizes and over longer durations form the basis for ongoing research into this area of immune system modulation.

Immunization for the prevention of periodontal disease may provide the greatest benefit to cost differential in the future, particularly for patients prone to developing early onset periodontal diseases or periodontitis associated with systemic disease. If the immune system can be modulated to prevent the emergence or destructive activities of pathogenic microorganisms in the periodontal pocket through active immunization or through passive immunization which would be indicated in immunocomprised patients, then it may be possible to prevent the rapid periodontal destruction which often accompanies these conditions.

V. SUMMARY AND CONCLUSIONS

Active immunization of *M. fascicularis* using the protease porphypain-2 in an attempt to immunologicaly interfere with the progression of experimentally-induced gingivitis and periodontitis produced no detectable adverse systemic response in the Nhp.

Immunization with porphypain-2 elicited a profound and sustained, specific immune response to both porphypain-2 and to the whole cells of *P. gingivalis*. This antibody response was predominantly of the IgG isotype. No significant alterations in the levels of IgM or IgA were induced by immunization.

Little change in antibody level was detected following ligature placement in the immunized animals.

Experimentally-induced gingivitis and periodontitis were accompanied by an increase in clinical measures of periodontal inflammation in both immunized and non-immunized Nhp.

Immunization had minimal impact on clinical parameters of periodontal disease including plaque scores, bleeding on probing, probing depths and clinical attachment levels.

Silk ligatures used to induce experimental periodontitis were not shown to significantly impact periodontal probing depth or clinical attachment level measurements in this Nhp model.

Active immunization was associated with an alteration in the microbial ecology in ligated sites. Fewer Gram-negative anaerobic bacterial species emerged significantly from baseline values in ligated sites in immunized Nhp compared to ligated sites in control Nhp during the progression of both experimental gingivitis and periodontitis.

Immunization was not able to prevent or suppress the emergence of *P. gingivalis* in ligated or non-ligated sites.

Ligature-induced periodontitis was accompanied by a significantly greater mean alveolar bone density loss in ligated sites within each group when compared to their respective unligated contralateral control sites. In this analysis, immunization was associated with a less significant degree of mean bone density loss compared to the non-immunized control group.

Further animal testing using different bacterial virulence factors as immunogens, greater sample sizes, and over longer durations may be done in an attempt to consider the feasibility of ultimately producing multi-component vaccines which incorporate a number of purified virulence factors from the most commonly implicated periodontal pathogens.

APPENDIX A

Modified Bleeding Index

Bleeding on Probing (within 20 seconds)

Clinical Response	Bleeding Score
No gingival bleeding	0
Gingival bleeding is pinpoint	1
Isolated bleeding point with a fine line	2
Interdental triangle fills with blood	3
Profuse bleeding, blood flows and fills the marginal sulcus	4
Spontaneous bleeding	5

APPENDIX B

Porphypain-2 Antigen Preparation

1. Added 3ml 0.2M bicarbonate buffer to one 500μg aliquot of 120 kDa protein.
2. Added 100μl 1M urea to second aliquot; added 5 drops 8M urea
3. Stored solutions at 4° C overnight
4. Prepared 0.4M Nh_4bicarbonate solution pH 8.0 in 8M urea. Spun down protein from previous day and saved buffer
5. Added 0.5ml Nh_4 bicarbonate/urea to each pellet; combined pellets and buffer and sonicated
6. Allowed to sit at room temperature for 1 hour
7. Resonicated
8. Allowed to sit at room temperature for 1 hour
9. Stored solutions at 4° C overnight
10. Resonicated and prepared as follows:

<u>Antigen</u>

1.65 ml antigen preparation + 5.1 ml ddH_20 = 6.75 ml IFA

<u>Placebo</u>

1.65 ml NH4bicarbonate in 8M urea + 5.1 ml ddH20 = 6.75 ml IFA

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VITA

Alan John Moritz was born in Oak Park, Illinois on March 7, 1960 to Hans K. and Christa M. Moritz. Dr. Moritz graduated from Lake Park High School, Roselle, Illinois in 1974. He subsequently attended Loyola University of Chicago and graduated in 1978 with a Bachelor of Science Degree in biology, honors, magna cum laude. He entered the University of Illinois College of Dentistry at Chicago and received his Doctor of Dental Surgery Degree in May 1986. After dental school, Dr. Moritz was commissioned as an officer in the United States Air Force and attended a one year general practice residency at Scott Air Force Base, Illinois. He subsequently served two consecutive overseas tours beginning in 1987 at Izmir Air Base, Turkey, and Yokota Air Base, Tokyo Japan. Returning to the United States in 1992, Dr. Moritz was assigned to Hill Air Force Base, Utah. In June 1994, Dr. Moritz entered the Post-doctoral Periodontics program at the University of Texas Health Science Center at San Antonio in conjunction with Wilford Hall Medical Center, Lackland Air Force Base, Texas. Dr. Moritz was married to Betsy Cruz on August 4, 1985; they have two children, Stephan Hans (age 6) and Julia Rosa (age 3).